MULTIFUNCTIONAL CROSS-LINKED HEMOGLOBIN CONJUGATES FOR PROTECTION OF PANCREATIC BETA CELLS

by

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ABSTRACT

The isolation of beta-islets from the pancreas and their microencapsulation in a polymeric matrix render them susceptible to hypoxic and hypoxia-induced free radical stresses, causing enhanced apoptosis *in vitro* and at the site of transplantation. This research project focused on low p50 hemoglobin (Hb) conjugates with the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), for Hb's protection from free radicals and from the combined oxidative and hypoxic stress for beta-islets. This dissertation is an account of the enhanced cell viability of beta-cells for an advancement toward a biohybrid artificial pancreas.

A poly(ethylene glycol) cross-linker used in an optimized Hb:PEG molar ratio (1:10) dramatically reduced methemoglobin formation. The addition of antioxidant enzymes with hemoglobin (Hb-SOD-CAT) inhibited methemoglobin formation during storage at 4 $^{\circ}$C for a month and also when subjected to external oxidative stress. The size of conjugates obtained by cross-linking was also optimal for coencapsulation with beta-cells, thus preventing the diffusional loss of Hb conjugates from the microcapsules.

The addition of poly(ethylene glycol) cross-linked Hb-SOD-CAT conjugates with low p50 characteristics and antioxidant properties showed enhanced protective effects on pancreatic cells (RINm5F cells) when incubated

in hypoxic (1% oxygen) conditions, as higher oxygen amounts are released in hypoxic condition. Combined hypoxic and free radical (hypoxia-induced) stress conditions are encountered during islet transplantation. RINm5F cells that had been incubated in hypoxia and challenged with oxidants (hydrogen peroxide and superoxide anion) showed low intracellular free radical activity on addition when optimized Hb-SOD-CAT conjugates was added. These optimized Hb-SOD-CAT conjugates also help maintain glucose-induced insulin secretion from pancreatic beta-islets in combined oxidative and hypoxic stress experiments.

In summary, the potential of Hb-SOD-CAT conjugates to reduce the dysfunction of beta-islets associated with free radical and hypoxic stress has been demonstrated. It is likely that antioxidant enzymes will play a significant role in the protection of hemoglobin in these conjugates and consequently will enhance cell viability. Consequently, the use of Hb-SOD-CAT conjugates in a biohybrid artificial pancreas consisting of an oxygen carrier, antioxidants, and pancreatic beta-islets in microcapsules has the potential to provide a better solution for long-term insulin secretion.

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ABBREVIATIONS

AO	Acridine orange
BAP	Bio-Artificial pancreas
CAT	Catalase
DCC	N,N′-Dicyclohexylcarbodiimide
DCF	Dichlorofluorescein
DCFH	Dichlorodihydrofluorescein
Hb	Hemoglobin
Hb-Hb	Cross-linked hemoglobin with poly(ethylene glycol)
Hb-SOD-CAT	Cross-linked hemoglobin with SOD and CAT by poly(ethylene glycol)
HBOCs	Hemoglobin based oxygen carriers
H2DCF-DA	2′, 7′-dichlorodihydrofluorescein diacetate
HIF-1	Hypoxia induced factor-1
H_2O_2	Hydrogen Peroxide
IDDM	Insulin-dependent diabetes mellitus
Met Hb	Methemoglobin
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
mU/mL	Milli units per milliliter

NHS	N-Hydroxysuccinimide
NOD	Non-obese diabetic mouse
$O_2^{\cdot -}$	Superoxide anion
OEC	Oxygen hemoglobin equilibrium curves
PEG	Poly(ethylene glycol)
pO_2	Partial oxygen pressure
PI	Propidium iodide
p50	Oxygen partial pressure at which hemoglobin is 50%saturatedwith O_2
RIA	^{125}I-Insulin radioimmunoassay
ROS	Reactive oxygen species
SEC	Size exclusion chromatography
SOD	Superoxide dismutase
XO	Xanthine oxidase

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1. INTRODUCTION

1.1 Physiology of the pancreas

1.1.1 Pancreas

The human pancreas contains approximately one million beta islets in the islets of Langerhans, the regions of the pancreas containing endocrine cells that distribute insulin throughout the exocrine tissues. Comprising 1% to 2% of the total mass of the pancreas, the islet of Langerhans contain four different cells types: beta cells, which produce insulin and decrease blood glucose levels; alpha cells, which produce glucagon and raise blood glucose levels; delta cells, which secrete somatostatin to inhibit the endocrine activity of the pancreas; and pancreatic polypeptide producing (PP) cells, which inhibit the exocrine process (*1*).

1.1.2 Insulin secretion and regulation

Although insulin is only produced by beta cells, insulin receptors are widely distributed on most types of cells (*2*). When the blood glucose level is elevated after digestion, insulin is released from the beta cells to convert glucose to glycogen via glycogenesis, which returns the elevated blood glucose level to a normal level. In the event of a drop in the normal blood glucose level, glucagon will be released by alpha cells to increase the blood glucose level by converting glycogen into glucose via glycogenolysis, a process mediated by the liver (*3*). Beta cells secrete insulin in response to exogenous glucose stimulation. On sensing elevated glucose, beta cells begin insulin synthesis by activation of two main receptor molecules, Glut2 and Hexokinase (*4*). Glucose enters through the

Glut2 receptor, which is phosphorylated by Glucokinase. Glut2 expression changes on the beta cell and responds to insulin synthesis based on whether the physiological state is diabetic or hyperglycemic. Insulin mRNA is translated into proinsulin, which contains the A and B chains of insulin linked by a C-peptide, a structure that helps align the disulfide bridges. Proinsulin is further processed in the maturing granule to insulin with the removal of the C-peptide by the endopeptidases. The insulin molecule binds coordinately with zinc (Zn) to form hexamers (*2, 5*). The synthesized insulin is transported in the blood, where it decreases elevated blood glucose levels by transporting glucose into the cells.

The insulin receptor on the cells belongs to the tyrosine kinase receptor family. When insulin binds to the insulin receptor, the substrate protein insulin receptor substrate 1 (IRS-1) is phosphorylated by tyrosine kinase. This leads to an increase in the number of glucose transporter (Glut4) molecules on the outer membrane of glucose-responsive cells, resulting in increased uptake of glucose from blood into the tissues. This glucose is further converted into glycogen and fatty acids or metabolized to produce energy (*5*)**.**

1.2 Diabetes

Diabetes mellitus or diabetes is a metabolic disorder state in which blood sugar levels are elevated either because the body does not produce sufficient insulin or because the insulin produced is not utilized effectively by body cells. Diabetes is categorized into Type 1 diabetes (*6*), also known as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes, a condition in which the

body fails to produce sufficient insulin and Type 2 diabetes (*7*), a condition in which insulin resistance and/or beta-cell failure results in insufficient insulin production.

1.2.1 Epidemiology of diabetes

The American Diabetes Association (ADA) estimates that nearly 24 million children and adults or 7.8% of the total population currently suffer from diabetes in the United States, of whom approximately 12 million are men and 11.5 million are women. According to death certificate data, diabetes is the seventh leading cause of death in the United States, in which an ADA estimated 1.6 million new cases are diagnosed every year. Of the 24 million Americans who suffer from diabetes, 5% to 10% suffer from Type 1 diabetes, a complex disease requiring lifelong insulin therapy. It has been estimated that approximately US$174 billion were spent on diabetic patients in 2007 (*8*). The World Health Organization (WHO) estimates that 285 million (6.4%) of the world population of 7 billion lives with diabetes, of whom 50.8 and 43.2 million live in India and China, respectively (*9*), and of whom approximately 480,000 are children suffering from Type 1 diabetes, the more common form among young children (*10*). WHO estimates that if the financial burden of diabetes is expressed in International Dollars (ID) that take into account differences in purchasing power, the global financial burden of diabetes was 418 ID billion in 2010, and projects it to increase to 561 ID billion by 2030 (*9*).

Apart from the enormous financial burden that diabetes imposes, current treatment of the disease requires the administration of multiple daily insulin injections, which is associated with poor patient compliance. Type 1 diabetes is also associated with long-term complications, such as diabetic retinopathy, cardiovascular disease, nephropathy, neuropathy, and atherosclerosis. The current ADA recommendation is to maintain glycosylated Hb (HbA1c) levels below 7% to control the disease and its associated complications (*8*).

1.2.2 Pathophysiology of Type 1 diabetes

Type 1 diabetes is an autoimmune disorder characterized by the complete absence of insulin production resulting from attacks on the beta cells in the islets of Langerhans by the body's immune system, which renders the beta cells incapable of insulin production (*6*). In most Type 1 diabetics, more than 90% of their islets have been destroyed by T-cells (*11-13*). This disease may be caused by genetic factors; environmental factors (*14*), such as viruses (*15*); vitamin deficiencies (*16*); chemically induced oxidative stress (streptozotocin) (*17*); and/or other unknown factors. The pathogenesis of beta-cell apoptosis involves the development of spontaneous auto-antigens and associated cellular (macrophage, dendritic cell, B lymphocyte, and T-cell) involvement (*18*). The main mechanism of beta-cell apoptosis in Type 1 diabetes is cytokine-mediated apoptosis, as opposed to high-glucose associated toxicity, which is the main mechanism in Type 2 diabetes (*19*). Other mechanisms, if any, are not very well understood. The disease is currently treated by injection of replacement insulin

obtained from exogenous sources and careful monitoring of blood glucose levels throughout the life of the patient.

1.3 Type 1 diabetes treatments and associated problems

Several approaches are currently being investigated to treat hyperglycemia and related complications of Type 1 diabetes (Table 1.1). The major limitation of the most commonly used approach, the administration of exogenous insulin, is patient inconvenience in monitoring and maintaining proper glucose levels (*20*). Although the use of insulin pumps is an attractive alternative, it requires improvement, and still requires the self-monitoring of blood glucose to maintain metabolic control. Although they have been improved, sensors and continuous glucose monitors are still inconvenient to use (*21, 22*).

An ideal solution is to replace the nonfunctioning endocrine part of the pancreas with healthy tissue that can produce insulin. However, doing so requires substitution of the entire pancreas with a healthy pancreas from a suitable donor. Such transplantation is infeasible for several reasons, including immune rejection of the transplanted organ, the limited off-shelf availability of a suitable pancreatic organ donor, and complications associated with the surgical procedure (*23*). At present, transplantation is limited to patients with severe insulin-dependent diabetes and associated complications, and is usually associated with simultaneous kidney transplant from the same donor, as pancreas-only transplantation tends to result in serious side effects on kidney functioning. The most common problems associated with transplantation include

Table 1.1: Current treatment options for Type 1 diabetes

Exogenous Insulin	**Insulin Pumps**	**Whole Pancreas Transplantation**	**Beta-islet Transplantation**
Advantages			
Controls blood glucose levels	Controls glucose fluctuations	Safe for patients with no cardiovascular complications	Minor surgery
Disadvantages			
Daily injections for life	Bulky pump and/ or connections	Major surgery with possible side effects	Islet scarcity & Immune suppression
Difficult to manage glucose fluctuations	Weight gain	Immune suppression; kidney damage	Duration of cell survival

thrombosis, infection, pancreatitis, and serious immune rejection, apart from donor scarcity and the need for a complex surgical procedure (*23*).

An alternative practical solution to entire organ transplantation is transplantation of isolated islets or islets from different sources (autogenic, allogenic, xenogenic, and/or artificial cell lines), which requires minimal and less invasive surgery. One of the many problems with transplanted islets from nonhuman sources/donors, that of rejection by the immune system, can be avoided by using either immunosuppressive drugs such as sirolimus (*24*), a cellular antiproliferative agent or an antibody-mediated immune modulating approach, such as anti-CD3 mAb (*25, 26*).

1.3.1 Clinical trial: Islet transplantation with immunosuppressive regimen

A research group in Edmonton, Alberta that achieved normal insulin production and normoglycemia in many patients via islet transplantation according to the Edmonton protocol (*27*) has conducted the most successful clinical trial of islet transplantation. In this trial, the team transplanted 9,000 allogenic islets/kg into the liver of each patient, a procedure that required 2 to 4 healthy human pancreas to treat a single patient (*28*), and that was found to have restored normoglycemia of greater than 50% one year after transplantation. However, the need for 2 to 3 donors per islet transplant makes this approach infeasible, and the need for immunosuppression throughout the lifetime may make it unsuitable for maintaining long-term normoglycemia (*27-31*).

1.3.2 Islet scarcity

To some extent, the problem of islet scarcity can be resolved using new approaches, including islet regeneration therapy (*32, 33*), in which beta cells arise primarily from preexisting beta cells, and the generation of islets from stem cells (*34, 35*). In the genetic engineering approach, the insulin-producing gene is inserted into non-insulin-producing cells or into cells that are sensitive to glucose (*36*). However, the genetic engineering approach faces several limitations. Even though islet scarcity can be resolved to some extent using the above approaches, islet transplantation requires immunosuppression throughout the transplanted xenogenic islet lifetime to prevent immune rejection, making them

very difficult for patients unless they are combined with other technologies that circumvent the need for immunosuppression. A feasible alternative approach that reduces immune rejection is to transplant only the insulin-producing beta cells. One such approach that is currently being pursued is the bioartifical pancreas (BAP) approach, which uses isolated islets (*37-39*) and biocompatible polymers to prevent the entry of modulators of immune response. Although islet transplantation has been demonstrated to have some success in clinical settings, it requires improvement to reduce the incidence of hypoxia and free-radical-induced islet death. Prevention of hypoxia-induced-islet apoptosis is a key factor in maintaining long-term insulin secretion and successful islet transplantation.

1.3.3 Islet transplantation without immunosuppression: The BAP

A bioengineering approach that has significant advantages over the above approaches, BAP employs islet encapsulation with islet-protecting biological molecules, such as oxygen (O_2) carriers, and signaling molecules, such as glucagon-like peptide 1, using a physical barrier. This semipermeable biocompatible physical layer allows the diffusion of secreted insulin across the physical barrier and assists in the mass transport of other essential nutrients by diffusion and the removal of waste materials from the bioartificial pancreas. The material barrier around the islets prevents the triggering of the immune response by inhibiting attack by immune cells and by preventing a foreign body response, and could thus potentially prevent graft rejection. This approach may also circumvent the need for a histocompatile donor and does not require major

surgery, as the implant can be transplanted in the liver through the portal system or in the peritoneal cavity (*37-39*). Although many researchers have demonstrated successful islet transplantation without immunosuppression, a significant number of hurdles are yet to be overcome. One such problem is the extremely large size of the implant, as a large number of microencapsulated islets (15,000-20,000 islets/kg of body weight) are required to achieve normoglycemia (*40*).

1.3.4 Immunoisolation devices

Based on their geometry and configuration, BAP devices are generally divided into the two main categories of 1) macrodevices/capsules and 2) microcapsules. Although both categories are being evaluated, microcapsules are currently more popular, as they are easy to retrieve and help keep the endocrine tissue together. However, these devices have the drawbacks of tissue loading, large size, potential for fibroblast growth inside the tissue, and lack of scalability to humans (*23, 41*). A vascular prosthesis in which islets are seeded within a special immunoselective chamber has shown some success in a dog model (*42, 43*), and is currently popular. However, its success in humans may be limited because of the risk of thrombosis and vascular accidents if the device fails.

In BAP systems using microcapsules, the materials used for the physical barriers are primarily polymers, such as alginate, poly-L-lysine, and poly-L-ornithine. The most currently successful approach is achieved by microencapsulation of islets using alginate and poly-L-lysine polymers. These

microcapsules, which can be transplanted both extravascularly and intravascularly (*41*), have better diffusion capacity compared to macrocapsules, are easy to transplant, and require minimal surgery. The encapsulation of individual islets inhibits fibroblast growth (*44, 45*), but the islets are difficult to retrieve. It has been shown that the ideal capsules range was around 700 μm in size and has no imperfections in their shape (*46*). Polycations such as poly-L-lysine bind to the alginate molecules and induce ionic complex formation at the capsule surface, which decreases the porosity of the membrane. The molecular cutoff diameter of the immunoisolation device can be controlled with the content of poly-L-lysine (*47*).

Current methodologies, including electrostatic droplet generation (*48*), gradient centrifugation (*49*), and photopolymerization (*50*), can decrease the size of microcapsules and the implant volume. However, the fundamental problems associated with islet viability, apoptosis, and necrosis inside the capsules by hypoxia and free-radical damage must be addressed when immunoisolation techniques are used.

1.4 Cell death

Cell death within the body occurs by different intracellular and extracellular pathways ways and is mediated by different mechanisms. Programmed cell death can be apoptotic (Type 1) or autophagic (Type 2). In apoptotic cell death, which is characterized by mitochondrial dysfunction followed by activation of caspase-dependent pathways, cell membrane integrity is lost, followed by cell

shrinkage, nuclear disintegration, and DNA fragmentation (*51*). Autophagic cell death due to the recycling of cytoplasm and the elimination of defective organelles, processes that regulate cellular homeostasis, is characterized by the appearance of double-membrane-containing vacuoles in the cytoplasm and the fusion of autophagosomes with lysosomes (*52*). Regulatory proteins, such as caspases and proteins of the bcl-2 family are primarily involved in cell death, and the activation of death receptors, such as the TNF receptor-1 and the Fas receptor, on the cell surface will cause cell death (*52*). Cell death can also occur by other apoptotic-mediated means due to cell stress or trauma, whether caspase-independent or necrotic in nature.

1.4.1 Cell adaptations and death under hypoxia

During hypoxic conditions, cells react to a demand for O_2 that exceeds availability by changing their metabolism, such as by increasing respiration via new blood vessel recruitment and erythropoiesis, the production of new red blood cells (RBCs). However, if chronic hypoxic conditions persist, cells change their means of respiration from aerobic to anaerobic, which reduces cell energy usage and decreases cell proliferation to meet the O_2 availability. Under normoxia, cells convert glucose into pyruvate, which is catalyzed in a respiratory reaction to produce ATP. However, under hypoxia, pyruvate is converted into lactate due to the shift in metabolism to an anaerobic pathway. Specifically, acidification of the microenvironment due to lactic acid production and reduced dispersion or removal of carbon dioxide (CO_2) from the cellular environment causes a change

in cellular metabolism. To some extent, cells adapt to this situation by up-regulation of carbonic anhydrase enzyme, which converts CO_2 to carbonic acid and allows cells to adapt to the acidic microenvironment (*53*).

Hypoxia causes autophagy through different mechanisms and depends on hypoxia-inducible factor (HIF). Researchers have found that if O_2 levels fall below 5%, the alpha subunit of the protein stabilizes, combines with the beta unit, and is expressed and elevated (*54*). Researchers have also found that additional pathways activated under severe hypoxic conditions can cause autophagy (*55*). Additional proteins, such as DJ-1, can also cause cell death by an unknown mechanism, as can PDGFR-dependent autocrine stimulation, stress-mediated metabolic stimulation, and up-regulation of endoplasmic reticulum (*55, 56*). In research examining isolated cell cultures, hypoxia has been found to cause significant changes at the metabolic level by activation or inhibition of the various enzymes and transporters discussed above, with the duration, severity, and type of cells studied determining the type of pathways activated or inhibited, which cannot be consistently defined (*57*).

1.4.2 Cell adaptations and death in a free-radical environment

Under conditions of normal O_2 levels, electron flow (1 to 2%) throughout the mitochondrial respiratory chain leads to the production of reactive oxygen species (ROS), which serve as signaling molecules in regulating cellular activity by controlling the oxidized targets (*58*), and are thus essential in cellular metabolism. Although it is logical to assume that free-radical generation is lower

in hypoxic conditions due to lower levels of O_2, the ROS level has been found to increase in such conditions (*57*). Specifically, it has been demonstrated that hypoxia triggers mitochondrial ROS, which have a significant role in HIF-1α stabilization during hypoxia (*57*). It has also been found that the sensing of low O_2 levels and the stabilization of free-radical-mediated HIF-1aplha are mediated by the mitochondrial respiratory chain (*57*). ROS are known to be produced during cellular hypoxia within different cells types, such as pulmonary artery smooth muscle cells and cardiomyocytes that produce increased ROS, which are usually detected by oxidation of fluorescent probes and are involved in cell death (*59*).

1.5 Islet hypoxia and possible approaches to its prevention

Islets are prone to hypoxic conditions during the stages of isolation (*60*), culturing (*60*), encapsulation (*61*), and transplantation (*62*). Most islets are susceptible to hypoxic conditions because of the enzymatic separation of islets, and thus the loss of blood vasculature or the network of blood capillaries around islets. Islets depend on surrounding blood vessels for their blood supply *in vivo* and after isolation from the pancreas and also on dissolved O_2 *in vitro* (*61, 63*). The location of beta cells—at the core of the islets of Langerhans, which are themselves aggregates of cells—increases the difficulty of supplying O_2 to the core in devascularized conditions (*61, 64, 65*). Dionne et al. have shown that low O_2 tension has a significant impact on the insulin secretion of islets, reporting that insulin secretion decreased to 50% of normal when islets were cultured at a

low O_2 pressure (pO_2) of 27 mm Hg and to 2% at a very low pO_2 of 5 mm Hg compared to islets cultured at a normal pO_2 of 142 mm Hg (5% CO_2, 95% air) (*66*). Scherzenmeir et al. obtained similar results, reporting a decrease in insulin secretion at pO_2 levels below 12 to 20 mm Hg (*67*).

Microencapsulation isolates islets from the surrounding environment and increases the diffusion barrier for O_2 transport, with O_2 availability and the quantity of dissolved O_2 varying according to the site of islet transplantation, whether the peritoneal cavity, liver, or kidneys (*61, 68, 69*). Researchers have found that vascular structures may be developed around nonencapsulated islets transplanted within the first few weeks (*70*). However, such development is inhibited when islets are encapsulated using microencapsulation techniques, and causes chronic hypoxic stress in encapsulated conditions and allows islet O_2 transport to occur only through diffusion from the surrounding milieu instead of from direct supply from the bloodstream (*61, 68*).

Finding the O_2 pressure within the islets to be 20 to 23 mm Hg before isolation (*71*), researchers have identified hypoxic stress in the peritoneal cavity as the cause of decreased insulin secretion, and thus the cause of progressive failure of transplanted islets (*72*). The mean pO_2 in isolated islets has been found to be approximately 40 mm Hg before transplantation and to decrease by a mean of approximately 5 mm Hg after transplantation of islets, irrespective of transplant site (kidney, liver, or spleen), even though the sites have significant differences in their blood perfusion in both diabetic or nondiabetic subjects. This finding demonstrates the importance of improving O_2 supply to the islets to enhance

their viability in transplantation conditions to avoid issues related to poor oxygenation or revascularization (*62*).

To prevent hypoxia-related islets apoptosis, O_2 carriers may be used to continuously provide O_2 to the islets. Colton et al. designed an electrochemical O_2 generator that continuously generates O_2 by the hydrolysis of water into hydrogen and O_2, and encapsulated an insulin-secreting cell line that demonstrated increased viability (*73*). However, this approach may not be suitable for microencapsulation because it involves a bulky power supply system and presents *in vivo* biocompatibility issues regarding transplantation, particularly the generation of O2 bubbles. A second approach, the use of highly O_2-dissolving perfluorocarbon emulsions in the encapsulated systems (*74, 75*), poses the challenges of lack of emulsion stability and lack of biocompatibility of the fluorocarbons, leading to concerns regarding islet toxicity. A third approach is the use of hypoxia-resistant islets isolated from a teleost Tilapia, which tolerates lower levels of O_2 pressure than do mammalian islets, for encapsulation. However, the abundance of the islets and their long-term viability in encapsulated and transplanted conditions may also be a concern in this approach (*76*).

1.5.1 Hb as an O_2-carrying agent

Hemoglobin (Hb) is a natural O_2-carrier molecule that can be easily isolated from RBCs, in which it is the primary O_2 carrier molecule, supplying more than 98% of the entire O_2 supply to the tissues. Nonfunctional RBCs are the typical sources of cell-free isolated Hb. Hb in RBCs transports O_2 from the

lungs to the tissue capillaries, where it exchanges it for CO_2. Hb is a polypeptide with a molecular weight of ~64.5 KDa composed of two alpha chains complexed with an iron molecule that pair with two beta chains to form a tetrameric complex with four binding sites for O_2 molecules (*77, 78*). Hb loads O_2 molecules at higher pO_2 conditions in the lungs and offloads O_2 molecules at lower pO_2 levels. Even the sites of islet transplantation have low pO_2 levels, which may help Hb molecules release O_2 molecules at low pressures. The typical O_2 equilibrium curve of Hb assumes a sigmodal shape with cooperative binding sites for O_2 molecules, i.e., the binding of one O_2 molecule to Hb produces a cooperative effect that leads to the binding of the other three oxygen molecules (*79-81*).

The objective in the design of most artificial O_2 carriers is to create Hb with O_2-carrying properties similar to those of Hb inside RBCs. However, this is an ambitious task because the Hb environment and the pressure within RBCs are different when Hb is outside the RBCs. Moreover, Hb exhibits altered properties and instability after isolation, having been shown to have an altered polypeptide complex and altered O_2-binding and release properties when outside RBCs. Hb-O_2 binding properties are also affected by pH, the presence of carbon monoxide (CO), and temperature. According to the Bohr Effect, at low pH, protons bind to a protein and change its conformation between deoxy and oxy-state. As CO has a high binding affinity to Hb, it competes with O_2 and decreases O_2-binding capacity. Methemoglobin is a form of Hb with no O_2-binding capacity that is used to prevent oxidation of Hb and allow it function as an effective O_2 carrier (*5*). Using commercially bought (bovine) isolated Hb with a high (75%)

methemoglobin content, Scherzenmeir et al. demonstrated improved functionality and viability of the cultured islets (*67, 82*).

1.5.2 Rationale for Hb modification

Isolated Hb is preferred over RBCs as an O_2 carrier because it can be stored for a longer term and leads to fewer immunogenic problems when performing blood-type matching (*83*). However, using isolated Hb poses the problem of the tetrameric dissociation of Hb to dimers (32 kDa) that undergo glomerular filtration, causing nephrotoxicity (*84*). The use of heme iron in dimeric form, which undergoes more rapid oxidation compared to heme iron in tetrameric form, leads to a series of events that results in the generation of reactive O_2 and nitrogen species, and thus results in oxidative damage (*85*).

1.5.3 Intra- and intermolecular cross-linking

To overcome the problems associated with their use, stroma-free isolated Hb molecules are modified using different approaches to increase their stability and molecular size. One such approach, intra- and intermolecular cross-linking, is a process by which isolated Hb molecules are cross-linked to form large molecular conjugates(*86*). This cross-linking can be specific or nonspecific, depending on the conjugating agent and the type of conjugation. Controlling the rate of reaction and the size of the conjugate is more difficult using nonspecific cross-linking agents than is using specific cross-linking agents (*86*).

Intramolecular cross-linking of Hb primarily assists in stabilizing the tetrameric conformation and thus preventing excretion by the kidneys. The intramolecular cross-linker DBBF (bis(3,5 dibromosalicyl)fumarate) cross-links two lysine-99 residues(*88, 89*). Intermolecular Hb cross-linking addresses the problem of dimers and increases the Hb size by cross-linking intermolecularly; one such cross-linking agent is O-raffinose (*90*). One of the most extensively studied cross-linking agents is glutaraldehyde, which reacts with alpha amine groups and terminal alpha amino groups on lysine residues and cross-links both intra- and intermolecularly (*91*). However, all these approaches have failed in clinical trials, primarily because their use led to nitric oxide (NO)-associated hypertension (Table 1.2) (*87*). Less common approaches used in Hb stabilization are encapsulation (*92*); site-directed mutagenesis to improve specific Hb properties, such as resistance to peroxidases and auto-oxidation (*93*); and the use of recombinant technology to design Hb with properties similar to native Hb, such as the retention of physiologically relevant O_2 and heme affinity (*94*).

1.5.4 Chemistry of PEG-based cross-linking

Poly(ethylene glycol) (PEG) is a linear or branched polymerized ether with hydroxyl end groups with the following general structure:

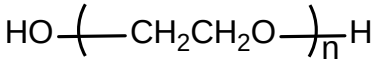
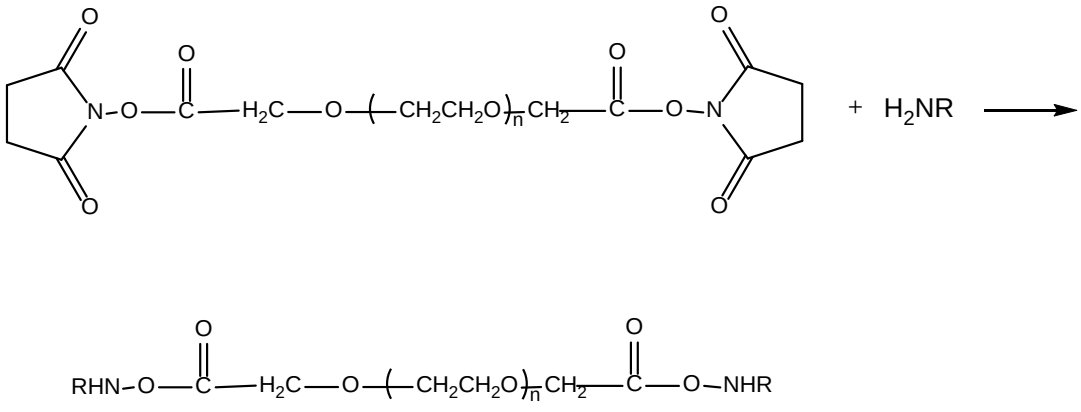


Table 1.2: Chemical modifications and adverse events of hemoglobin based oxygen carriers evaluated in phase II/III clinical trials, modified and adapted from (*87*).

Product	**Chemical modification**	**Adverse events**
Hemopure® (Biopure)	Glutaraldehyde polymerized	Death, Hypertension Acute renal failure
Polyheme® (Northfield Labs)	Pyridoxal-5'-phosphate cross-linked and glutaraldehyde polymerized	Death Hypertension
PHP® (Apex Bioscience)	Pyridoxal-5'-phosphate cross-linked and polyethylene conjugated	Death Hypertension
Hemospan® (Sangart)	Maleimide-polyethylene glycol conjugated	Acute renal failure, Death Hypertension
Hemolink® (Hemolink)	Raffinose cross-linked and polymerized	Acute renal failure Hypertension
HemAssit® (Baxter)	Bis-3,5-dibromosalicyl fumarate cross-linked	Acute renal failure Hypertension

PEG is activated at one or both ends for conjugation and functionalized based on the reactive group on the protein or petide. Common reactive amino acids on the protein include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine, N-terminal amino group acids, and C-terminal carboxylic acid. The most common route for the PEG conjugation of proteins has been activation of PEG with functional groups suitable for reaction with lysine. N-terminal amino acid groups are routinely used for specific and nonspecific PEG conjugation by activating PEG with suitable functionalization agents, including acids such as COOH. One of the most prevalent amino acids, lysine, accounts for 10% of all amino acids in the amino acid sequencing of protein (*95*).

PEG can be broadly classified into the categories of alkylating and acylating PEG. This study used an acylating method to randomly conjugate Hb with antioxidant enzymes. Active PEG is in the form of the hydroxysuccinimidyl esters (-OSu) structure below.



The distance between the active ester can vary up to four methylene units, with the length of the X units in the chain having a significant effect on the reactivity and hydrolysis in water. The SuO-CO-X-$(CH_2CH_2O)_n$-O-X-CO-OSu used in this conjugation had a hydrolysis half-life of 0.75 hours (*96*).

Pegylation is an approach used to stabilize or cross-link Hb molecules (*97, 98*). Polyethylene glycol, the biocompatible polymer, is commercially available in different molecular weights and branches for conjugation with many types of molecules, depending on the research need (*99, 100*). PEG is an attractive agent because MP4, Hemospan ® chemically modified with monofunctional PEG-activated with a terminal maleimide group maintains its effectiveness in hypoxic tissue oxygenation by preventing autoregulatory vasoconstriction (*101*). This property arises from its favorable properties, such as a large molecular excluded volume, high viscosity, high oncotic pressure (exerted by blood proteins by pulling water into circulatory system), and high O_2 affinity (*102*). PEG-based Hb has recently been demonstrated to carry O_2 in microcirculation and release more than 95% of the saturated O_2 into the tissues (*103*).

1.5.5 Importance of low-p50 (high-affinity) Hb under hypoxia

When compared to that within the lungs, the site of Hb oxygenation, O_2 pressure *in vivo* is low at tissue and cellular sites (Figure 1.1) (*104*). This difference in O_2 pressure leads the Hb molecule to efficiently release O_2 from organs to tissues at the cellular level (*105*). When cells are prone to hypoxic stress their increased demand for O_2 requires the use of an O_2 carrier that can

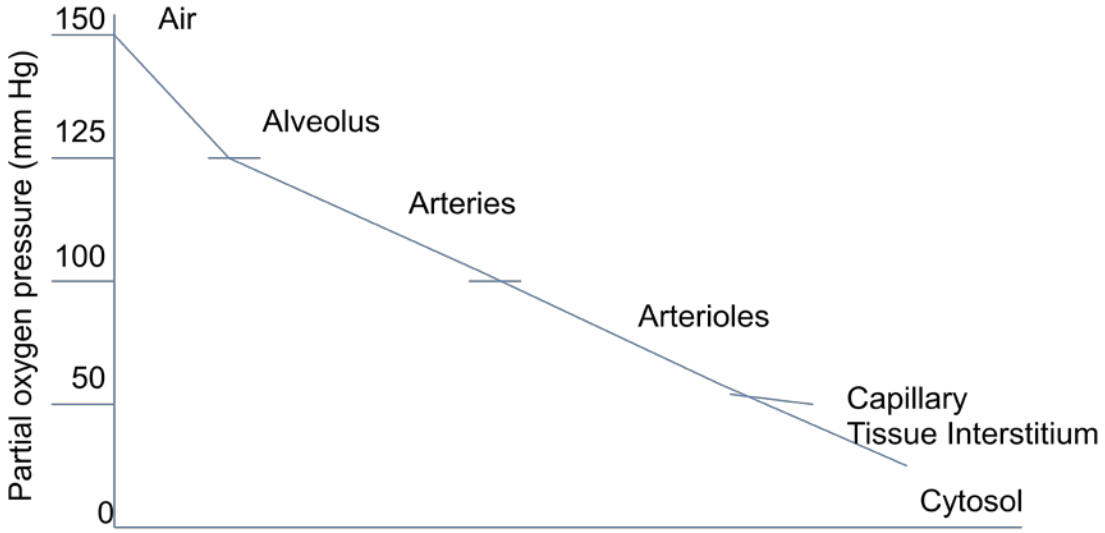


Figure 1.1. Representation of pO_2 differences from the airways to the cytosol, modified and adapted from (*104*).

release a greater quantity of O_2 (*106*). One such carrier would be a low-p50 Hb-based O_2 carrier that helps release relatively large amounts of O_2 at low pressures under hypoxic conditions (*107*). Low-p50 Hb, which has a high affinity for O_2, binds O_2 in circulation and allows O_2 release in only O_2-deficient hypoxic conditions, protecting cells from hypoxic stress. By the differential expression of Hb macromolecules of higher and lower O_2 affinity (*108*), the crustacean species *Daphnia magna* has developed a tolerance to hypoxia that has allowed it to adapt to extreme environments with limited O_2 availability.

1.5.6 O_2 carriers and buffering capacity

O_2 carriers carry and release O_2 passively by equilibrating with the surrounding pO2, as described by the Hill equation (*109*),

$$\text{Fractional Saturation} = \frac{(pO_2)^n}{(p50)^n + (pO_2)^n}$$

where *n* is the cooperativity and p50 is the pO_2 saturation level at which saturation is equal to half the normal level. Outside the binding range, the O_2 carrier is either fully oxygenated above a partial level of O_2 saturation or fully deoxygenated below the lower limit, and any change in the surrounding partial O_2 pressure will not change its O_2 saturation. Hb-based O_2 carriers are not O_2 pumps, and will only equilibrate with the surrounding partial O_2 pressure by replacing the consumed O_2 saturation. Hb-based O_2 carriers are not O_2 pumps, and will only equilibrate with the surrounding partial O_2 pressure by replacing the consumed O_2.

Thus, the buffering capacity of O_2 carriers, which is the ratio of change in partial O_2 saturation to partial O_2 pressure, is determined by the sensitivity of the carrier to the gradient changes of surrounding pO_2 (*109*). For example, Hb saturation varies between 10% and 70% when pO_2 increases from 7 to 40 mm Hg (~1% to 6% O_2) for p50 of 24.5 mm Hg and *n* = 1.75 but between 78% and 95% in myoglobin for 2 mm of p50 mm Hg and *n* = 1, indicating that O_2 carriers act as better buffering agents near their p50 value and demonstrating the greater effectiveness of Hb than myoglobin for hypoxic transplantation conditions of 1% to 6% O_2. Due to the absence of cooperativity, myoglobin has a lower buffering capacity than Hb.

During increased O_2 demand by cells, cell-free Hb assists in the formation or re-occupation of replaced O_2 by facilitating O_2 transport by diffusion through plasma. The rate of diffusion (translational) in O_2 carriers through liquid has been shown to be inversely proportional to the viscosity of the medium and the size of the carrier, and thus isolated Hb-based O_2 carriers are able to replace O_2 more rapidly than are RBCs (*109*). In cell-bound O_2 transport, the normal physiological decrease in O_2 from the lung to the tissue level helps deliver higher amounts of O_2. In cell-free Hb, low p50 and facilitated diffusion assist in O_2 re-saturation and delivery in hypoxic conditions (*110*).

Cell-free Hb increases the solubility of O_2 and decreases the resistance to O_2 diffusion by constantly exchanging O_2 between the Hb and O_2 dissolved in media. O_2 delivery in normoxic tissues has been shown to be relatively insensitive to p50 values for O_2 delivery. However, values below 15 Torr can increase O_2 unloading in hypoxic conditions when Hb p50 is lowered (high-affinity). Increased O_2 cooperative binding increases O_2 delivery, even in cases of substantially reduced delivery. In one study, cell-free sebacyl cross-linked Hb supported intestinal function under hypoxic conditions, while an equivalent quantity of Hb inside RBCs provided poor support (*110*).

1.6 Effect of ROS (reactive oxygen species) on islets and Hb

Free radicals are species or molecular fragments that have one or more unpaired electrons. Potential endogenous sources of free radicals include mitochondria; cytochrome p450; microsomes; and peroxisomes of cells, such as

neutrophils, eosinophils, and macrophages. Exogenous processes or substrates, such as radiation; carcinogens; chlorinated compounds; and redox metal ions, such as iron, generate ROS either directly or indirectly (*111, 112*). The most common ROS are the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical (OH·), and H_2O_2, which are generated intracellularly by different subcellular processes, and eventually cause lipid peroxidation and cell death (*113*). Superoxide anions are formed when an O_2 molecule acquires an additional electron, leaving it with an unpaired electron. Hb that undergoes oxidation by peroxide and auto-oxidation by endogenously produced oxidants produces methemoglobin, which has an oxidation state of +3 and cannot effectively bind to O_2. The iron catalyzation that occurs in Hb by superoxide according to the Fenton reaction and the Haber-Weiss reaction can produce damaging short-lived hydroxyl radicals (Table 1.3) (*112*). Furthermore, the methemoglobin produced releases heme more readily than does its ferrous counterpart, increasing the potential for iron-mediated free-radical toxicity (*114*). In RBCs, methemoglobin is maintained at a level less than 1% of total Hb (*115*) by the intrinsic reductive mechanisms of the Embden-Meyeroff pathway and the pentose phosphate pathway (*117, 118*). Post transplantation, islets are prone to stress, and undergo apoptosis due to the presence of free radicals (*119*), NO (*120*), interleukin-1 (*121*), interferon-γ, tissue necrosis factor-alpha (*122*), and other ROS that are released due to the activation of immune cells and macrophages or because of glucose-catalyzed oxidative stress (*123*). In addition, insulin-secreting cells, such as pancreatic islets and insulinoma cells, express low levels of antioxidant enzymes, such as

Table 1.3: Free-iron-catalyzed oxidation reactions on Hb, modified and adapted from (*116*).

Iron catalyzed oxidation reactions on Hemoglobin

Fenton reaction

$$H_2O_2 + Fe\ (+II) \rightarrow Fe\ (+III) + OH^- + OH^\cdot$$

$$O_2^{\cdot -} + Fe\ (+III) \rightarrow Fe\ (+II) + O_2$$

Haber-Weiss reaction

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + OH^\cdot$$

Oxidation of oxyHb by hydrogen peroxide

$$HbFe\ (+II)\ O_2 + H_2O_2 \rightarrow HbFe\ (+IV) =O + H_2O + O_2$$

Auto-oxidation of Hb

$$HbFe\ (+II)\ O_2 \rightarrow HbFe\ (+III) + O_2^{\cdot -}$$

Direct reduction of oxygen by ferrous iron

$$Fe\ (+II) + O_2 \rightarrow Fe\ (+III) + O_2^{\cdot -}$$

Hydroxyl radical generation from heme-free iron and hydrogen peroxide

$$Fe\ (+II) + H_2O_2 \rightarrow Fe\ (+III) + OH^- + OH^\cdot$$

superoxide dismutase (SOD) and catalase (CAT), that assist in the removal of superoxide anions and H_2O_2, making islets prone to free-radical-induced oxidative damage (*124, 125*).

Several genetic and chemical modifications have been proposed as means of enhancing the resistance of insulin-secreting cells to oxidative stress. Recently, hemeoxygenase-1 (HO-1) (*126*), an anti-oxidant enzyme expressed by chemical treatment of islets, was reported to protect islets from radical scavenging and aid in apoptosis prevention. Protection of islets from oxidative stress is a potential approach to preventing the early graft failure of encapsulated

islets, with a coencapsulation approach using cross-linked Hb-Hb having been found effective in preventing an NO attack *in vitro* (*100*).

1.7 Antioxidant enzymes

The most efficient enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase. Nonenzymatic antioxidants include vitamin C; vitamin E; carotenoids; thiol antioxidants, such as glutathione, thioredoxin, and lipoic acid; natural flavonoids; and melatonin, a hormonal product of the pineal gland (*112*). Researchers have used many antioxidant molecules, such as vitamin C, heme oxygenase, vitamin E, SOD, CAT, and many other chemically synthesized compounds, to help prevent free-radical-mediated cell death (*113*).

1.7.1 Superoxide dismutase

Appearing in one of three forms, SOD (32 kDa) is a group of metal-containing enzymes that catalyze the reduction of a superoxide radical to H_2O_2 and O_2 (Table 1.4).

Two forms of SOD, copper/zinc SOD (Cu/Zn-SOD), which is present in the cytoplasm, and manganese SOD (Mn-SOD), which is present in the mitochondria, are intracellular, while the third form, extracellular SOD (EC-SOD), is, as indicated by its name, extracellular, and contains Cu and Zn at its active site. This study used bovine erythrocyte SOD, a type of intracellular Cu/Zn-SOD that is characterized as a dimeric protein with two identical subunits of 151 amino

Table 1.4: Mechanism of SOD and CAT protection, modified and adapted from (*127*).

Superoxide dismutase	**Catalase**
Reaction $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$	Reaction $2H_2O_2 \rightarrow 2H_2O + O_2$
Reaction Mechanism	Reaction Mechanism
$SOD\text{-}Cu^{+2} + O_2^{\cdot-} \rightarrow SOD\text{-}Cu^{+} + O_2$ $SOD\text{-}Cu^{+} + O_2^{\cdot-} + 2H^+ \rightarrow SOD\text{-}Cu^{+2} + H_2O_2$	$CAT\text{-}Fe^{+3} + H_2O_2 \rightarrow CAT\text{-}[Fe^{+4} = O]^{\cdot}$ $CAT\text{-}[Fe^{+4} = O]^{\cdot} + H_2O_2 \rightarrow CAT\text{-}Fe^{+3} + H_2O + ½\ O_2$

acids, with each subunit containing 10 free lysines available for modification. Cu/Zn-SOD contains seven conserved His residues that play an important role in enzymatic activity and an “electrostatic loop” that contains four highly conserved charged residues (Lys120, Glu130, Glu131, and Lys134) that promote the catalytic action by attracting and directing superoxide anions toward the Cu site (*127*).

1.7.2 Catalase

CAT is an intracellular tetrameric enzyme with four subunits, each bearing one ferric protoporphyrin in the prosthetic group (*128*), that catalyzes the breakdown of H_2O_2 to H_2O and O_2 (*112*). Regarding the enzyme catalysis, which is shown in Table 1.4, cross-linking has demonstrated that there is no loss in the activity of the enzymes, thus indicating that the conjugation may not be affecting

the enzyme's active site (*129*). The CAT active site structure includes a heme molecule; distal His-54 and Asn-127, which are involved in H_2O_2 binding; and proximal Tyr-337, which binds to the proximal heme iron ligand and His-341(*130, 131*).

1.8 Summary of current challenges in using the BAP

- Islet scarcity
- Lack of encapsulating membrane compatibility
- Complete immunoisolation
- Lack of cell viability
- Lack of oxygen supply (hypoxic stress)
- Free radical stress

The remaining sections of this work focus on addressing the last two problems.

1.9 Proposed research strategy

Previous studies (PEG-conjugated Hb with SOD and CAT) found that encapsulation of PEG cross-linked Hb increased the insulin secretion of islets by increasing the O_2 supply (*132*). However, insulin secretion was sustained for only about three months, both *in vivo* and *in vitro* (*99, 132*). Even when Hb can supply O_2 to the encapsulated islets, it is prone to oxidation by conversion of Hb to methemoglobin. To prevent this deleterious oxidation reaction and thus enhance O_2 delivery to tissues, an effective Hb protection mechanism is required. The cross-linking of two cellular antioxidant molecules inherently present in the body,

SOD and CAT (*133, 134*), with Hb has been proposed as means of overcoming the dual problems of Hb oxidation and free-radical-mediated islet apoptosis. SOD and CAT are abundantly present in RBCs, where they serve as the primary antioxidants in protecting Hb from oxidative damage. As they work together, both enzymes must be microencapsulated to provide for effective protection of islets (Figure 1.2). SOD has been shown to decrease the Hb oxidative process by 31%, CAT to decrease it by 53%, and SOD and CAT together to decrease it by 76% (*135, 136*). Hypoxic conditions near the islets, such as that created by oxygen pressure below 40 mmHg at an implantation site, lead to a decrease in pH, decreasing the affinity of Hb for O_2 and release a large quantity of O_2 or unload O_2 as pO_2. An antioxidant-enzyme approach using low p50 PEG-based O_2-carriers has been proposed to enhance unloading of O_2 in large quantities below the p50 of the carrier under hypoxic (low O_2) conditions.The results obtained from this proposed research approach will assist in determining the capacity of O_2-carrying conjugates to deliver O_2 to the islets at implantation sites under conditions of hypoxia and the resulting hypoxia- induced free-radical environment. This approach has been shown to be partially successful in previous studies using only Hb (*99, 137*). This study attempted to confirm previous findings regarding the capacity of conjugates with low p50 to supply O_2, as well as investigate the added protection provided by the addition of antioxidant enzymes.

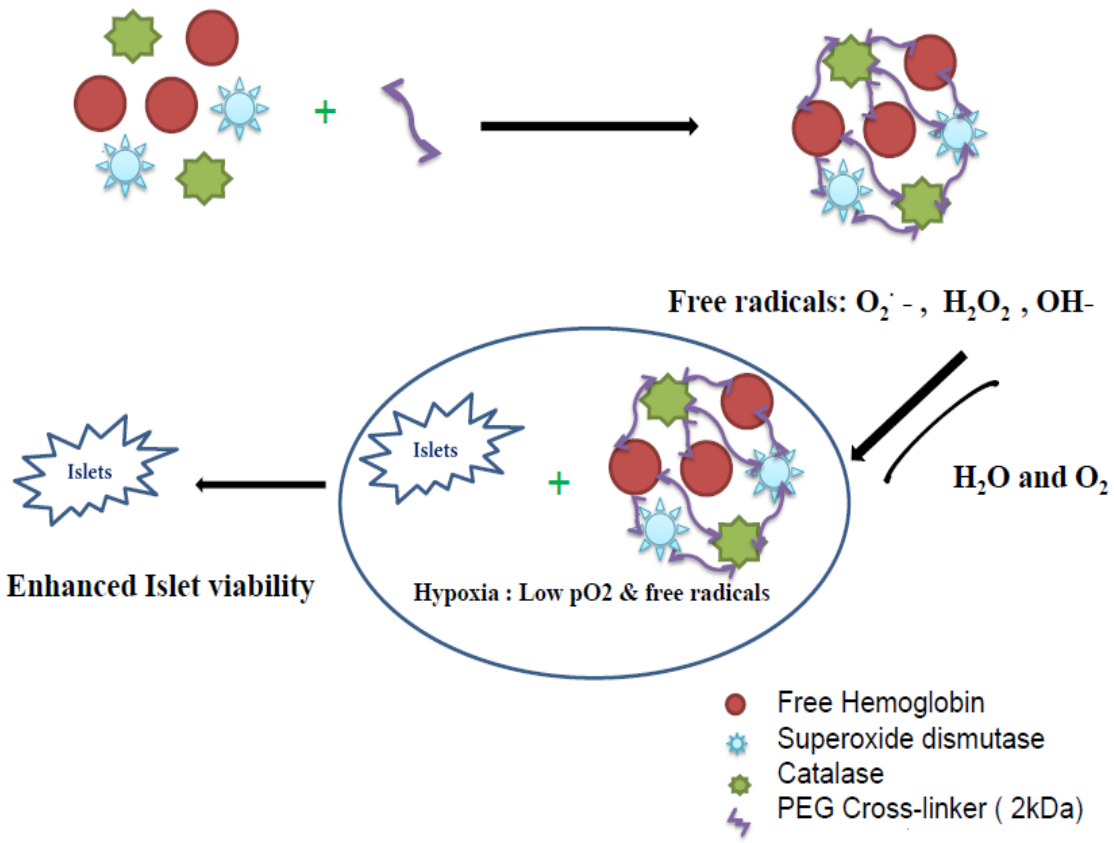


Figure 1.2: Schematic representation of research design

1.10 References

(1) Prado, C. L., Pugh-Bernard, A. E., Elghazi, L., Sosa-Pineda, B., and Sussel, L. (2004) Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci U S A 101*, 2924-9.

(2) Pierce, S. B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S. A., Buchman, A. R., Ferguson, K. C., Heller, J., Platt, D. M., Pasquinelli, A. A., Liu, L. X., Doberstein, S. K., and Ruvkun, G. (2001) Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. *Genes Dev 15*, 672-86.

(3) Heppner, K. M., Habegger, K. M., Day, J., Pfluger, P. T., Perez-Tilve, D., Ward, B., Gelfanov, V., Woods, S. C., DiMarchi, R., and Tschop, M.

(2010) Glucagon regulation of energy metabolism. *Physiol Behav 100*, 545-8.

(4) Matschinsky, F. M. (1996) Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. *Diabetes 45*, 223-41.

(5) Leibiger, I. B., Leibiger, B., Moede, T., and Berggren, P. O. (1998) Exocytosis of insulin promotes insulin gene transcription via the insulin receptor/PI-3 kinase/p70 s6 kinase and CaM kinase pathways. *Mol Cell 1*, 933-8.

(6) Eisenbarth, G. S. (2007) Update in type 1 diabetes. *J Clin Endocrinol Metab 92*, 2403-7.

(7) Tfayli, H., and Arslanian, S. (2009) Pathophysiology of type 2 diabetes mellitus in youth: the evolving chameleon. *Arq Bras Endocrinol Metabol 53*, 165-74.

(8) ADA. (2010) in *Diabetes Statistics.*

(9) WorldDiabetesFoundation. (2010).

(10) DiabetesAtlas. (2010) http://www.diabetesatlas.org/.

(11) Efrat, S. (2001) Cell therapy approaches for the treatment of diabetes. *Curr Opin Investig Drugs 2*, 639-42.

(12) Efrat, S. (2002) Cell replacement therapy for type 1 diabetes. *Trends Mol Med 8*, 334-39.

(13) Peshavaria, M., and Pang, K. (2000) Manipulation of pancreatic stem cells for cell replacement therapy. *Diabetes Technol Ther 2*, 453-60.

(14) Yoon, J. W., Kim, C. J., Pak, C. Y., and McArthur, R. G. (1987) Effects of environmental factors on the development of insulin-dependent diabetes mellitus. *Clin Invest Med 10*, 457-69.

(15) Yoon, J. W. (1990) The role of viruses and environmental factors in the induction of diabetes. *Curr Top Microbiol Immunol 164*, 95-123.

(16) al-Zuhair, H., and Mohamed, H. E. (1998) Vitamin C attenuation of the development of type I diabetes mellitus by interferon-alpha. *Pharmacol Res 38*, 59-64.

(17) Van Dyke, K., Jabbour, N., Hoeldtke, R., Van Dyke, C., and Van Dyke, M. (2010) Oxidative/nitrosative stresses trigger type I diabetes: preventable in

streptozotocin rats and detectable in human disease. *Ann N Y Acad Sci 1203*, 138-45.

(18) Yoon, J. W., and Jun, H. S. (2001) Cellular and molecular pathogenic mechanisms of insulin-dependent diabetes mellitus. *Ann N Y Acad Sci 928*, 200-11.

(19) Cnop, M., Welsh, N., Jonas, J. C., Jorns, A., Lenzen, S., and Eizirik, D. L. (2005) Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes 54 Suppl 2*, S97-107.

(20) Brunton, S. (2008) Insulin delivery systems: reducing barriers to insulin therapy and advancing diabetes mellitus treatment. *Am J Med 121*, S35-41.

(21) Shalitin, S., and Phillip, M. (2006) Closing the loop: combining insulin pumps and glucose sensors in children with type 1 diabetes mellitus. *Pediatr Diabetes 7 Suppl 4*, 45-9.

(22) Battelino, T. (2006) Risk and benefits of continuous subcutaneous insulin infusion (CSII) treatment in school children and adolescents. *Pediatr Diabetes 7 Suppl 4*, 20-4.

(23) Narang, A. S., and Mahato, R. I. (2006) Biological and biomaterial approaches for improved islet transplantation. *Pharmacol Rev 58*, 194-243.

(24) Reffet, S., and Thivolet, C. (2006) [Immunology of pancreatic islet transplantation.]. *Diabetes Metab 32*, 523-526.

(25) Young, D. L., Ramanujan, S., Kreuwel, H. T., Whiting, C. C., Gadkar, K. G., and Shoda, L. K. (2006) Mechanisms mediating anti-CD3 antibody efficacy: insights from a mathematical model of type 1 diabetes. *Ann N Y Acad Sci 1079*, 369-73.

(26) Sherry, N. A., Kushner, J. A., Glandt, M., Kitamura, T., Brillantes, A. M., and Herold, K. C. (2006) Effects of autoimmunity and immune therapy on beta-cell turnover in type 1 diabetes. *Diabetes 55*, 3238-45.

(27) Ryan, E. A., Lakey, J. R., Rajotte, R. V., Korbutt, G. S., Kin, T., Imes, S., Rabinovitch, A., Elliott, J. F., Bigam, D., Kneteman, N. M., Warnock, G. L., Larsen, I., and Shapiro, A. M. (2001) Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes 50*, 710-9.

(28) Ryan, E. A., Lakey, J. R., Paty, B. W., Imes, S., Korbutt, G. S., Kneteman, N. M., Bigam, D., Rajotte, R. V., and Shapiro, A. M. (2002) Successful

islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes 51*, 2148-57.

(29) Truong, W., and Shapiro, A. M. (2006) Progress in islet transplantation in patients with type 1 diabetes mellitus. *Treat Endocrinol 5*, 147-58.

(30) Nanji, S. A., and Shapiro, A. M. (2006) Advances in pancreatic islet transplantation in humans. *Diabetes Obes Metab 8*, 15-25.

(31) Shapiro, A. M., Geng Hao, E., Lakey, J. R., Finegood, D. T., Rajotte, R. V., and Kneteman, N. M. (2002) Defining optimal immunosuppression for islet transplantation based on reduced diabetogenicity in canine islet autografts. *Transplantation 74*, 1522-8.

(32) Sia, C., and Homo-Delarche, F. (2004) Tolerance induction and endogenous regeneration of pancreatic beta-cells in established autoimmune diabetes. *Rev Diabet Stud 1*, 198-206.

(33) Banerjee, M., Kanitkar, M., and Bhonde, R. R. (2005) Approaches towards endogenous pancreatic regeneration. *Rev Diabet Stud 2*, 165-76.

(34) Zulewski, H. (2006) Stem cells with potential to generate insulin producing cells in man. *Swiss Med Wkly 136*, 647-54.

(35) Street, C. N., Rajotte, R. V., and Korbutt, G. S. (2003) Stem cells: a promising source of pancreatic islets for transplantation in type 1 diabetes. *Curr Top Dev Biol 58*, 111-36.

(36) Efrat, S. (1999) Genetically engineered pancreatic beta-cell lines for cell therapy of diabetes. *Ann N Y Acad Sci 875*, 286-93.

(37) Bae, Y. H. (2004) Caged pancreatic islet for IDDM. *Yonsei Med J 45 Suppl*, 56-60.

(38) Chae, S. Y., Kim, S. W., and Bae, Y. H. (2001) Bioactive polymers for biohybrid artificial pancreas. *J Drug Target 9*, 473-84.

(39) Hou, Q. P., and Bae, Y. H. (1999) Biohybrid artificial pancreas based on macrocapsule device. *Adv Drug Deliv Rev 35*, 271-287.

(40) Soon-Shiong, P., Feldman, E., Nelson, R., Heintz, R., Yao, Q., Yao, Z., Zheng, T., Merideth, N., Skjak-Braek, G., Espevik, T., and et al. (1993) Long-term reversal of diabetes by the injection of immunoprotected islets. *Proc Natl Acad Sci U S A 90*, 5843-7.

(41) Calafiore, R. (1998) Actual perspectives in biohybrid artificial pancreas for the therapy of type 1, insulin-dependent diabetes mellitus. *Diabetes Metab Rev 14*, 315-24.

(42) Maki, T., and Monaco, A. P. (1997) Porcine islet xenotransplantation utilizing a vascularized bioartificial pancreas. *Ann Transplant 2*, 69-71.

(43) Maki, T., Otsu, I., O'Neil, J. J., Dunleavy, K., Mullon, C. J., Solomon, B. A., and Monaco, A. P. (1996) Treatment of diabetes by xenogeneic islets without immunosuppression. Use of a vascularized bioartificial pancreas. *Diabetes 45*, 342-7.

(44) Dufrane, D., Goebbels, R. M., Saliez, A., Guiot, Y., and Gianello, P. (2006) Six-month survival of microencapsulated pig islets and alginate biocompatibility in primates: proof of concept. *Transplantation 81*, 1345-53.

(45) De Vos, P., De Haan, B., and Van Schilfgaarde, R. (1997) Effect of the alginate composition on the biocompatibility of alginate-polylysine microcapsules. *Biomaterials 18*, 273-8.

(46) De Vos, P., De Haan, B., Pater, J., and Van Schilfgaarde, R. (1996) Association between capsule diameter, adequacy of encapsulation, and survival of microencapsulated rat islet allografts. *Transplantation 62*, 893-9.

(47) Siebers, U., Horcher, A., Bretzel, R. G., Federlin, K., and Zekorn, T. (1997) Alginate-based microcapsules for immunoprotected islet transplantation. *Ann N Y Acad Sci 831*, 304-12.

(48) Krestow, M., Lum, Z. P., Tai, I. T., and Sun, A. (1991) Xenotransplantation of microencapsulated fetal rat islets. *Transplantation 51*, 651-5.

(49) Zekorn, T., Siebers, U., Horcher, A., Schnettler, R., Zimmermann, U., Bretzel, R. G., and Federlin, K. (1992) Alginate coating of islets of Langerhans: in vitro studies on a new method for microencapsulation for immuno-isolated transplantation. *Acta Diabetol 29*, 41-5.

(50) Sawhney, A. S., Pathak, C. P., and Hubbell, J. A. (1993) Interfacial photopolymerization of poly(ethylene glycol)-based hydrogels upon alginate-poly(l-lysine) microcapsules for enhanced biocompatibility. *Biomaterials 14*, 1008-16.

(51) Zimmermann, K. C., Bonzon, C., and Green, D. R. (2001) The machinery of programmed cell death. *Pharmacol Ther 92*, 57-70.

(52) Diaz, L. F., Chiong, M., Quest, A. F., Lavandero, S., and Stutzin, A. (2005) Mechanisms of cell death: molecular insights and therapeutic perspectives. *Cell Death Differ 12*, 1449-56.

(53) Cassavaugh, J., and Lounsbury, K. M. (2011) Hypoxia-mediated biological control. *J Cell Biochem 112*, 735-44.

(54) Chandel, N. S., and Budinger, G. R. (2007) The cellular basis for diverse responses to oxygen. *Free Radic Biol Med 42*, 165-74.

(55) Kroemer, G., Marino, G., and Levine, B. (2010) Autophagy and the integrated stress response. *Mol Cell 40*, 280-93.

(56) Mehrpour, M., Esclatine, A., Beau, I., and Codogno, P. (2010) Overview of macroautophagy regulation in mammalian cells. *Cell Res 20*, 748-62.

(57) Solaini, G., Baracca, A., Lenaz, G., and Sgarbi, G. (2010) Hypoxia and mitochondrial oxidative metabolism. *Biochim Biophys Acta 1797*, 1171-7.

(58) Scherz-Shouval, R., and Elazar, Z. (2011) Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem Sci 36*, 30-8.

(59) Li, C., and Jackson, R. M. (2002) Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am J Physiol Cell Physiol 282*, C227-41.

(60) Linn, T., Schmitz, J., Hauck-Schmalenberger, I., Lai, Y., Bretzel, R. G., Brandhorst, H., and Brandhorst, D. (2006) Ischaemia is linked to inflammation and induction of angiogenesis in pancreatic islets. *Clin Exp Immunol 144*, 179-87.

(61) de Groot, M., Schuurs, T. A., and van Schilfgaarde, R. (2004) Causes of limited survival of microencapsulated pancreatic islet grafts. *J Surg Res 121*, 141-50.

(62) Carlsson, P. O., Palm, F., Andersson, A., and Liss, P. (2001) Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. *Diabetes 50*, 489-95.

(63) Heinis, M., Simon, M. T., Ilc, K., Mazure, N. M., Pouyssegur, J., Scharfmann, R., and Duvillie, B. (2010) Oxygen tension regulates pancreatic beta-cell differentiation through hypoxia-inducible factor 1alpha. *Diabetes 59*, 662-9.

(64) Cantley, J., Grey, S. T., Maxwell, P. H., and Withers, D. J. (2010) The hypoxia response pathway and beta-cell function. *Diabetes Obes Metab 12 Suppl 2*, 159-67.

(65) Morini, S., Braun, M., Onori, P., Cicalese, L., Elias, G., Gaudio, E., and Rastellini, C. (2006) Morphological changes of isolated rat pancreatic islets: a structural, ultrastructural and morphometric study. *J Anat 209*, 381-92.

(66) Dionne, K. E., Colton, C. K., and Yarmush, M. L. (1989) Effect of oxygen on isolated pancreatic tissue. *ASAIO Trans 35*, 739-41.

(67) Schrezenmeir, J., Gero, L., Solhdju, M., Kirchgessner, J., Laue, C., Beyer, J., Stier, H., and Muller-Klieser, W. (1994) Relation between secretory function and oxygen supply in isolated islet organs. *Transplant Proc 26*, 809-13.

(68) Yang, H., O'Hali, W., Kearns, H., and Wright, J. R., Jr. (1997) Long-term function of fish islet xenografts in mice by alginate encapsulation. *Transplantation 64*, 28-32.

(69) Schneider, S., von Mach, M. A., Kraus, O., Kann, P., and Feilen, P. J. (2003) Intraportal transplantation of allogenic pancreatic islets encapsulated in barium alginate beads in diabetic rats. *Artif Organs 27*, 1053-6.

(70) Jansson, L., and Carlsson, P. O. (2002) Graft vascular function after transplantation of pancreatic islets. *Diabetologia 45*, 749-63.

(71) Carlsson, P. O., Liss, P., Andersson, A., and Jansson, L. (1998) Measurements of oxygen tension in native and transplanted rat pancreatic islets. *Diabetes 47*, 1027-32.

(72) De Vos, P., Vegter, D., De Haan, B. J., Strubbe, J. H., Bruggink, J. E., and Van Schilfgaarde, R. (1996) Kinetics of intraperitoneally infused insulin in rats. Functional implications for the bioartificial pancreas. *Diabetes 45*, 1102-7.

(73) Wu, H., Avgoustiniatos, E. S., Swette, L., Bonner-Weir, S., Weir, G. C., and Colton, C. K. (1999) In situ electrochemical oxygen generation with an immunoisolation device. *Ann N Y Acad Sci 875*, 105-25.

(74) Kin, T., Mirbolooki, M., Salehi, P., Tsukada, M., O'Gorman, D., Imes, S., Ryan, E. A., Shapiro, A. M., and Lakey, J. R. (2006) Islet isolation and transplantation outcomes of pancreas preserved with University of Wisconsin solution versus two-layer method using preoxygenated perfluorocarbon. *Transplantation 82*, 1286-90.

(75) Ramachandran, S., Desai, N. M., Goers, T. A., Benshoff, N., Olack, B., Shenoy, S., Jendrisak, M. D., Chapman, W. C., and Mohanakumar, T. (2006) Improved islet yields from pancreas preserved in perflurocarbon is via inhibition of apoptosis mediated by mitochondrial pathway. *Am J Transplant 6*, 1696-703.

(76) Wright, J. R., Jr., Yang, H., and Dooley, K. C. (1998) Tilapia--a source of hypoxia-resistant islet cells for encapsulation. *Cell Transplant 7*, 299-307.

(77) Hu, T., Li, D., Manjula, B. N., Brenowitz, M., Prabhakaran, M., and Acharya, S. A. (2009) PEGylation of Val-1(alpha) destabilizes the tetrameric structure of hemoglobin. *Biochemistry 48*, 608-16.

(78) Dragan, S. A., Olsen, K. W., Moore, E. G., and Fitch, A. (2008) Spectroelectrochemical study of hemoglobin A, alpha- and beta-fumarate crosslinked hemoglobins; implications to autoxidation reaction. *Bioelectrochemistry 73*, 55-63.

(79) Hare, G. M., Harrington, A., Liu, E., Wang, J. L., Baker, A. J., and Mazer, C. D. (2006) Effect of oxygen affinity and molecular weight of HBOCs on cerebral oxygenation and blood pressure in rats. *Can J Anaesth 53*, 1030-8.

(80) Sasagawa, K., Imai, K., and Kobayashi, M. (2006) Influence of allosteric effectors and temperature on oxygen binding properties and the Bohr effect of bovine hemoglobin. *Zoolog Sci 23*, 565-72.

(81) Grinberg, O. Y., Hou, H. G., Roche, M. A., Merlis, J., Grinberg, S. A., Khan, N., Swartz, H. M., and Dunn, J. F. (2005) Modeling of the response of p(t)O(2) in rat brain to changes in physiological parameters. *Adv Exp Med Biol 566*, 111-118.

(82) Schrezenmeir, J., Velten, F., and Beyer, J. (1994) Immobilized hemoglobin improves islet function and viability in the bioartificial pancreas in vitro and in vivo. *Transplant Proc 26*, 792-800.

(83) Zhu, X., Chu, W., Wang, T., Wang, F., Fan, D., Dan, N., and Chen, C. (2007) Variations in dominant antigen determinants of glutaraldehyde polymerized human, bovine and porcine hemoglobin. *Artif Cells Blood Substit Immobil Biotechnol 35*, 518-32.

(84) Malchesky, P. S., Takahashi, T., Iwasaki, K., Harasaki, H., and Nose, Y. (1990) Conjugated human hemoglobin as a physiological oxygen carrier--pyridoxalated hemoglobin polyoxyethylene conjugate (PHP). *Int J Artif Organs 13*, 442-50.

(85) Mozzarelli, A., Ronda, L., Faggiano, S., Bettati, S., and Bruno, S. (2010) Haemoglobin-based oxygen carriers: research and reality towards an alternative to blood transfusions. *Blood Transfus 8 Suppl 3*, s59-68.

(86) Harris, D. R., and Palmer, A. F. (2008) Modern cross-linking strategies for synthesizing acellular hemoglobin-based oxygen carriers. *Biotechnol Prog 24*, 1215-25.

(87) Buehler, P. W., D'Agnillo, F., and Schaer, D. J. (2010) Hemoglobin-based oxygen carriers: from mechanisms of toxicity and clearance to rational drug design. *Trends Mol Med 16*, 447-57.

(88) Nagababu, E., Ramasamy, S., Rifkind, J. M., Jia, Y., and Alayash, A. I. (2002) Site-specific cross-linking of human and bovine hemoglobins

differentially alters oxygen binding and redox side reactions producing rhombic heme and heme degradation. *Biochemistry 41*, 7407-15.

(89) D'Agnillo, F., and Alayash, A. I. (2000) Site-specific modifications and toxicity of blood substitutes. The case of diaspirin cross-linked hemoglobin. *Adv Drug Deliv Rev 40*, 199-212.

(90) Scatena, R., and Giardina, B. (2001) O-raffinose-polymerised haemoglobin. A biochemical and pharmacological profile of an oxygen carrier. *Expert Opin Biol Ther 1*, 121-7.

(91) Chang, T. M. (2004) Hemoglobin-based red blood cell substitutes. *Artif Organs 28*, 789-94.

(92) Zhao, J., Liu, C. S., Yuan, Y., Tao, X. Y., Shan, X. Q., Sheng, Y., and Wu, F. (2007) Preparation of hemoglobin-loaded nano-sized particles with porous structure as oxygen carriers. *Biomaterials 28*, 1414-22.

(93) Isarankura-Na-Ayudhya, C., Tansila, N., Worachartcheewan, A., Bulow, L., and Prachayasittikul, V. (2010) Biochemical and cellular investigation of Vitreoscilla hemoglobin (VHb) variants possessing efficient peroxidase activity. *J Microbiol Biotechnol 20*, 532-41.

(94) Bobofchak, K. M., Mito, T., Texel, S. J., Bellelli, A., Nemoto, M., Traystman, R. J., Koehler, R. C., Brinigar, W. S., and Fronticelli, C. (2003) A recombinant polymeric hemoglobin with conformational, functional, and physiological characteristics of an in vivo O2 transporter. *Am J Physiol Heart Circ Physiol 285*, H549-61.

(95) Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Adv Drug Deliv Rev 54*, 459-76.

(96) Veronese, F. M. (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials 22*, 405-17.

(97) Li, D., Hu, T., Manjula, B. N., and Acharya, S. A. (2009) Extension arm facilitated pegylation of alphaalpha-hemoglobin with modifications targeted exclusively to amino groups: functional and structural advantages of free Cys-93(beta) in the PEG-Hb adduct. *Bioconjug Chem 20*, 2062-70.

(98) Meng, F., Manjula, B. N., Tsai, A. G., Cabrales, P., Intaglietta, M., Smith, P. K., Prabhakaran, M., and Acharya, S. A. (2009) Hexa-thiocarbamoyl phenyl PEG5K Hb: vasoactivity and structure: influence of rigidity of the conjugation linkage on the PEGylation induced plasma expander-like solution properties of PEG-Hb adducts. *Protein J 28*, 199-212.

(99) Chae, S. Y., Kim, S. W., and Bae, Y. H. (2002) Effect of cross-linked hemoglobin on functionality and viability of microencapsulated pancreatic islets. *Tissue Eng 8*, 379-94.

(100) Chae, S. Y., Lee, M., Kim, S. W., and Bae, Y. H. (2004) Protection of insulin secreting cells from nitric oxide induced cellular damage by crosslinked hemoglobin. *Biomaterials 25*, 843-50.

(101) Vandegriff, K. D., and Winslow, R. M. (2009) Hemospan: design principles for a new class of oxygen therapeutic. *Artif Organs 33*, 133-8.

(102) Bjorkholm, M., Fagrell, B., Przybelski, R., Winslow, N., Young, M., and Winslow, R. M. (2005) A phase I single blind clinical trial of a new oxygen transport agent (MP4), human hemoglobin modified with maleimide-activated polyethylene glycol. *Haematologica 90*, 505-15.

(103) Cabrales, P., Meng, F., and Acharya, S. A. (2010) Tissue oxidative metabolism after extreme hemodilution with peg conjugated hemoglobin. *J Appl Physiol*.

(104) Ward, J. P. (2008) Oxygen sensors in context. *Biochim Biophys Acta 1777*, 1-14.

(105) Mik, E. G., Stap, J., Sinaasappel, M., Beek, J. F., Aten, J. A., van Leeuwen, T. G., and Ince, C. (2006) Mitochondrial PO2 measured by delayed fluorescence of endogenous protoporphyrin IX. *Nat Methods 3*, 939-45.

(106) Stein, J. C., and Ellsworth, M. L. (1993) Capillary oxygen transport during severe hypoxia: role of hemoglobin oxygen affinity. *J Appl Physiol 75*, 1601-7.

(107) Schumacker, P. T., Suggett, A. J., Wagner, P. D., and West, J. B. (1985) Role of hemoglobin P50 in O2 transport during normoxic and hypoxic exercise in the dog. *J Appl Physiol 59*, 749-57.

(108) Paul, R. J., Zeis, B., Lamkemeyer, T., Seidl, M., and Pirow, R. (2004) Control of oxygen transport in the microcrustacean Daphnia: regulation of haemoglobin expression as central mechanism of adaptation to different oxygen and temperature conditions. *Acta Physiol Scand 182*, 259-75.

(109) Bucci, E. (2009) Thermodynamic approach to oxygen delivery in vivo by natural and artificial oxygen carriers. *Biophys Chem 142*, 1-6.

(110) Koehler, R. C., Fronticelli, C., and Bucci, E. (2008) Insensitivity of cerebral oxygen transport to oxygen affinity of hemoglobin-based oxygen carriers. *Biochim Biophys Acta 1784*, 1387-94.

(111) Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., and Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol 39*, 44-84.

(112) Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., and Mazur, M. (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact 160*, 1-40.

(113) Bashan, N., Kovsan, J., Kachko, I., Ovadia, H., and Rudich, A. (2009) Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. *Physiol Rev 89*, 27-71.

(114) Chang, T. M. (2005) Therapeutic applications of polymeric artificial cells. *Nat Rev Drug Discov 4*, 221-35.

(115) Jaffe, E. R. (1981) Methemoglobin pathophysiology. *Prog Clin Biol Res 51*, 133-51.

(116) Linberg, R., Conover, C. D., Shum, K. L., and Shorr, R. G. (1998) Hemoglobin based oxygen carriers: how much methemoglobin is too much? *Artif Cells Blood Substit Immobil Biotechnol 26*, 133-48.

(117) Faivre, B., Menu, P., Labrude, P., and Vigneron, C. (1998) Hemoglobin autooxidation/oxidation mechanisms and methemoglobin prevention or reduction processes in the bloodstream. Literature review and outline of autooxidation reaction. *Artif Cells Blood Substit Immobil Biotechnol 26*, 17-26.

(118) Bewley, M. C., Marohnic, C. C., and Barber, M. J. (2001) The structure and biochemistry of NADH-dependent cytochrome b5 reductase are now consistent. *Biochemistry 40*, 13574-82.

(119) Rabinovitch, A. (1992) Free radicals as mediators of pancreatic islet beta-cell injury in autoimmune diabetes. *J Lab Clin Med 119*, 455-6.

(120) Kolb, H., and Kolb-Bachofen, V. (1992) Type 1 (insulin-dependent) diabetes mellitus and nitric oxide. *Diabetologia 35*, 796-7.

(121) Thibault, V., Terlain, B., and Ekindjian, O. G. (1993) [Interleukin-1 and regulation of glucose homeostasis]. *Pathol Biol (Paris) 41*, 178-86.

(122) Mandrup-Poulsen, T., Helqvist, S., Wogensen, L. D., Molvig, J., Pociot, F., Johannesen, J., and Nerup, J. (1990) Cytokine and free radicals as effector molecules in the destruction of pancreatic beta cells. *Curr Top Microbiol Immunol 164*, 169-93.

(123) Robertson, R., Zhou, H., Zhang, T., and Harmon, J. S. (2007) Chronic oxidative stress as a mechanism for glucose toxicity of the beta cell in type 2 diabetes. *Cell Biochem Biophys 48*, 139-46.

(124) Tiedge, M., Lortz, S., Munday, R., and Lenzen, S. (1998) Complementary action of antioxidant enzymes in the protection of bioengineered insulin-producing RINm5F cells against the toxicity of reactive oxygen species. *Diabetes 47*, 1578-85.

(125) Tiedge, M., Lortz, S., Drinkgern, J., and Lenzen, S. (1997) Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes 46*, 1733-42.

(126) Li, M., Peterson, S., Husney, D., Inaba, M., Guo, K., Kappas, A., Ikehara, S., and Abraham, N. G. (2007) Long-lasting expression of HO-1 delays progression of type I diabetes in NOD mice. *Cell Cycle 6*, 567-71.

(127) Agnillo, D. (1997), McGill University, Montreal.

(128) Hajdu, J., Wyss, S. R., and Aebi, H. (1977) Properties of human erythrocyte catalases after crosslinking with bifunctional reagents. Symmetry of the quaternary structure. *Eur J Biochem 80*, 199-207.

(129) D'Agnillo, F., and Chang, T. M. (1998) Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. *Nat Biotechnol 16*, 667-71.

(130) Gouet, P., Jouve, H. M., and Dideberg, O. (1995) Crystal structure of Proteus mirabilis PR catalase with and without bound NADPH. *J Mol Biol 249*, 933-54.

(131) Gouet, P., Jouve, H. M., Williams, P. A., Andersson, I., Andreoletti, P., Nussaume, L., and Hajdu, J. (1996) Ferryl intermediates of catalase captured by time-resolved Weissenberg crystallography and UV-VIS spectroscopy. *Nat Struct Biol 3*, 951-6.

(132) Chae, S. Y., Kim, Y. Y., Kim, S. W., and Bae, Y. H. (2004) Prolonged glucose normalization of streptozotocin-induced diabetic mice by transplantation of rat islets coencapsulated with crosslinked hemoglobin. *Transplantation 78*, 392-7.

(133) Powanda, D. D., and Chang, T. M. (2002) Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. *Artif Cells Blood Substit Immobil Biotechnol 30*, 23-37.

(134) Quebec, E. A., and Chang, T. M. (1995) Superoxide dismutase and catalase cross-linked to polyhemoglobin reduces methemoglobin formation in vitro. *Artif Cells Blood Substit Immobil Biotechnol 23*, 693-705.

(135) Mal, A., and Chatterjee, I. B. (1991) Mechanism of Auto-oxidation of oxyhemoglobin. *J. Biosci., 16*, 55.

(136) Mal, A., Nandi, A., and Chatterjee, I. B. (1991) Haemoglobin: A scavenger of superoxide radical. *J. Biosci., Vol. 16*.

(137) Avila, J. G., Wang, Y., Barbaro, B., Gangemi, A., Qi, M., Kuechle, J., Doubleday, N., Doubleday, M., Churchill, T., Salehi, P., Shapiro, J., Philipson, L. H., Benedetti, E., Lakey, J. R., and Oberholzer, J. (2006) Improved Outcomes in Islet Isolation and Transplantation by the Use of a Novel Hemoglobin-based O(2) Carrier. *Am J Transplant*.

2. SYNTHESIS AND CHARACTERIZATION OF HEMOGLOBIN CONJUGATES WITH ANTIOXIDANT ENZYMES VIA POLY(ETHYLENE GLYCOL) CROSS-LINKER

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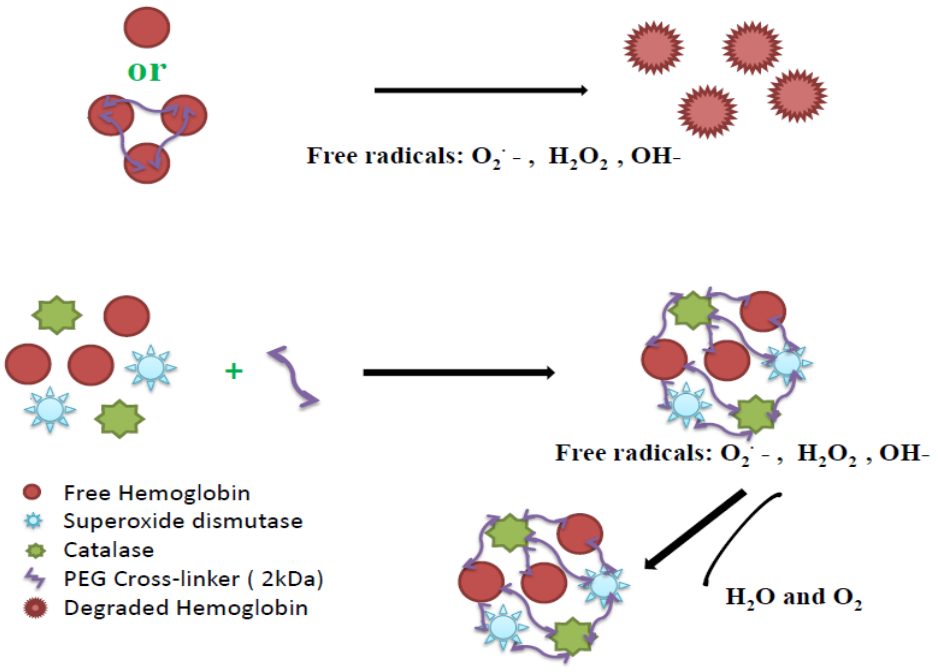
Nadithe, V., and Bae, Y. H. (2010) Synthesis and characterization of hemoglobin conjugates with antioxidant enzymes via poly(ethylene glycol) cross-linker (Hb-SOD-CAT) for protection from free radical stress. *Int J Biol Macromol 47*, 603-13. http://www.sciencedirect.com/science/journal/01418130

2.1 Abstract

Employing dicarboxymethylated poly(ethylene glycol) to conjugate Hb with antioxidant enzymes has been proposed for a means of providing enhanced protection to Hb against free radicals. This study examined the effectiveness of this conjugation process employing SDS-PAGE and SEC techniques to produce Hb-SOD-CAT conjugates of an estimated average molecular weight of 1000 kDa. When incubated with H_2O_2 (0.1 and 1mM) and xanthine (1mM)/xanthine oxidase (10 and 20mUnits/mL), the conjugates were found to retain greater than 70% to 90% of their original bioactivity. During the conjugation process and over one month of storage at 4 $^{\circ}$C, the antioxidant enzymes were found to minimize methemoglobin formation. Based on the results, the study concluded that optimized (1:10 Hb:PEG) cross-linked conjugates with antioxidant enzymes provide cells with protection from severe free-radical stresses (Scheme 2.1).



Scheme 2.1: Graphical Abstract

2.2 Introduction

Cell-free Hb has been widely investigated as an artificial O_2 carrier to treat conditions such as hemorrhagic shock (*1*), anemia (*2*), and ischemia (*3*) by means of blood transfusion. However, cell-free Hb is unstable, and the tetramer dissociation is known to cause nephrotoxicity (*4*). Vasoconstriction, another problem with using cell-free Hb, has been a primary reason for its failure as an O_2 carrier in many clinical trials (*5*). Fortunately, a number of different approaches, including cross-linking (*6*), polymerization (*7*), the use of recombinant technology (*8*), and encapsulation (*9, 10*), have shown very promising results in stabilizing tetrameric Hb. Stabilizing Hb by these approaches has been shown to help deliver O_2 efficiently in blood transfusions and other treatments under conditions of normal O_2 pressure within the body. However, most of these approaches only stabilize the alpha and beta tetramers of Hb, and do not offer much protection from auto-oxidation (*11*) or free radicals, such as hydroxyl and superoxide anions (*12*). Inside RBCs, high levels of antioxidant enzymes, such as SOD and CAT, assist in the protection of Hb from free-radical damage (*13*). To have a protective effect in the above oxidation conditions, an Hb-based O_2-carrier system with an antioxidant defense mechanism that can protect itself from both hypoxia-induced free radicals and environmental radical stressors to preserve Hb O_2-carrying capability is required (*14*).

Although glutarladehyde cross-linking has been shown to stabilize hemoglobin, increasing the glutaraldehyde-to-hemoglobin molar ratio above 40:1 increases the methemoglobin content (*15*). The addition of antioxidant enzymes

has been demonstrated to reduce oxidation (*16*), but the release of glutaraldehyde by degradation of the polymeric link and its associated toxicity remains a significant concern (*17*). The promotion of vasoconstriction by the NO-scavenging of glutaraldehyde-cross-linked products from the vasculature is also a serious concern (*18, 19*). It is therefore highly desirable to design a cross-linking reagent that is of low cytotoxicity and high biocompatibility.

One approach, the use of PEG-based modified polymers, has shown to reduce hypertensive responses and the NO-scavenging effect, and thus inhibit vasoconstriction. These effects may be attributed to the beneficial effects associated with PEG polymers, such as an increase in molecular size and hydrodynamic volume (*20*). Extensive literature has described approaches using PEG in proteins for pharmaceutical applications as useful and safe, as well as appropriate for conjugating Hb. The molecular weight of PEG used in the conjugates is important, as the use of Hb modified by PEG of low molecular weight has been shown to decrease the vasoconstrictive effect to a greater extent than the use of PEG of high molecular weight (*21*). PEG-modified Hb has been shown to offer the unique advantages of low cooperativity and high O_2 affinity or low p50 (*20, 22*). It has also been reported that cysteine modification of Hb using PEG-malemide chemistry increases Hb auto-oxidation and enhances oxidation by PEG chains in the presence of H_2O_2 (*23*).

Previous research by the authors of this paper has shown that cross-linked Hb improves the functionality of encapsulated isolated pancreatic beta cells in transplanted diabetic mice by maintaining O_2 supply to encapsulated cells

(*24, 25*). However, the viability of Hb is limited due to its eventual conversion to methemoglobin and the free-radical damage that is sustains from auto-oxidation. To overcome these problems in a manner in accordance with the most recent literature, this study investigated the use of polyethylene glycol, SOD, and CAT to conjugate the available amino groups (lysine, arginine, asparagine, glutamine, and histidine) on Hb. Although polymerization can stabilize Hb, the addition of SOD and CAT may be a better strategy, provided the enzymatic actions inhibit the conversion of Hb into methemoglobin. This particular conjugate design may also help protect Hb from oxidation during chemical conjugation, auto-oxidation, and free radical stress during storage in a size suitable for co-encapsulation with isolated islets for cell transplantation.

2.3 Experimental section

2.3.1 Material

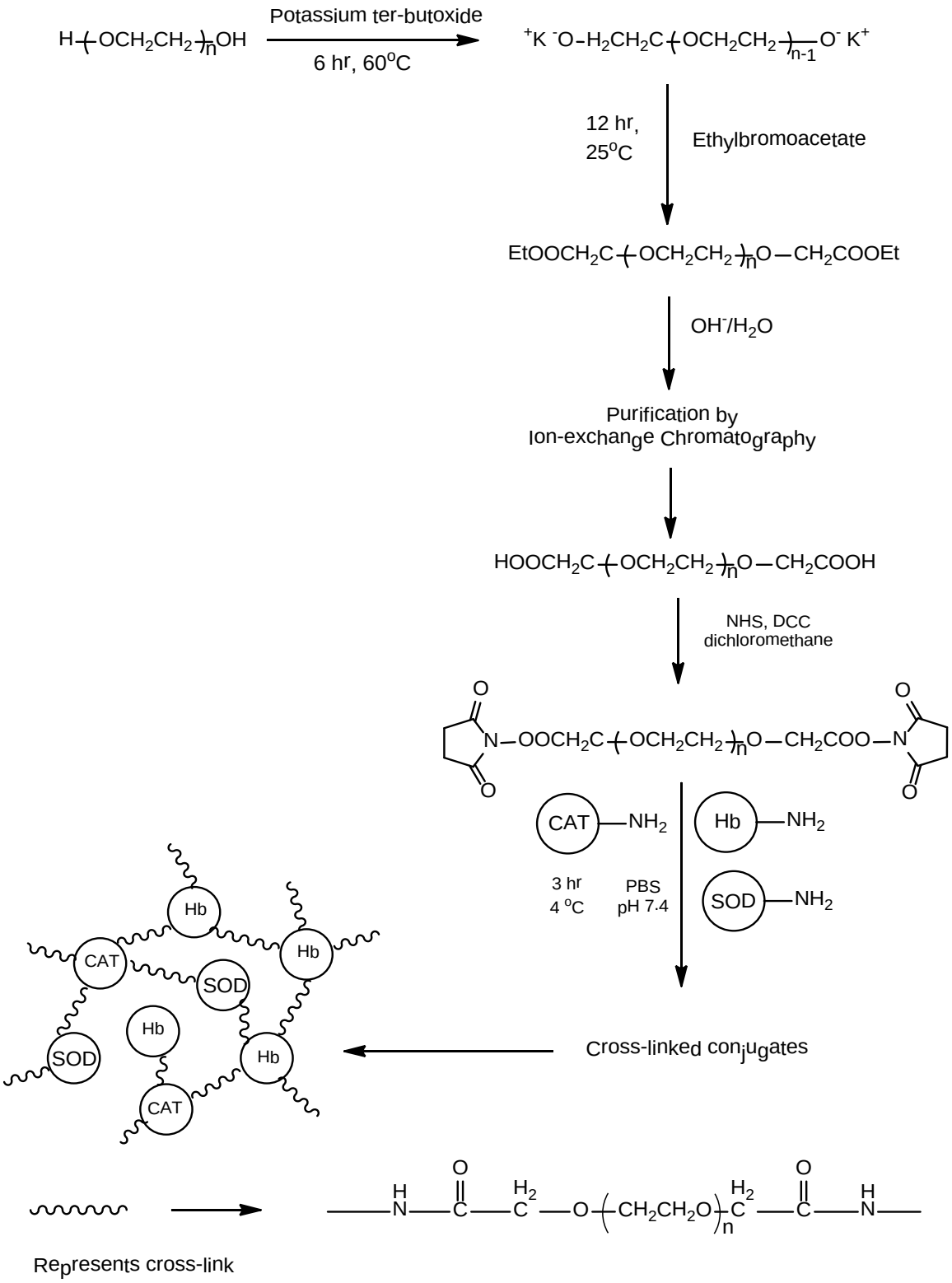
Freshly pooled bovine RBCs were purchased from Innovative Research (Novi, MI IC100-0410). Polyethylene glycol (molecular weight: 2 kDa), superoxide dismutase from bovine erythrocytes (SOD-S7571), bovine liver catalase (CAT-C40), tertiary butoxide, ethyl bromoacetate, N-Hydroxy-succinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), hydrogen peroxide (H_2O_2), xanthine, and xanthine oxidase (XO) were purchased from Sigma Chemical Co. (MO, USA). Dialysis membranes were purchased from spectrum labs (Rancho Dominguez, CA). Amicon Ultra-15 Centrifugal Filter Units, 50 and 100 kDa, were purchased from Millipore Corporation (Billerica, MA).

2.3.2 Hb isolation

Briefly, freshly withdrawn bovine RBCs were washed twice with normal saline solution by centrifuging at 2500 rpm for 20min to isolate the Hb. The Hb was extracted from the washed RBCs by mixing the substrate with 4:1 water and dichloromethane and shaking it for 5min. Released Hb that partitioned into the aqueous phase was collected by further centrifuging at 2500 rpm for 20min. The process was repeated twice and the isolated Hb centrifuged to remove any remaining organic solvent. The isolated Hb was dialyzed for 48h using a 50 kDa dialysis membrane, then transferred to a 100 kDa dialysis tube and dialyzed for 24h such that only pure Hb diffused out, which was further concentrated using Amicon membranes. The purity of the Hb was verified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified using Drabkin's method (*24*).

2.3.3 PEG modification

PEG (2 kDa) was customized for conjugation by employing previously reported methods (*24, 26, 27*). Briefly, the hydroxyl group of PEG was converted to ethyl-protected carboxymethylated PEG using potassium tertiary butoxide and ethyl bromoacetate. Hydrolysis of the product using sodium hydroxide resulted in a mixture of carboxylic (mono and di) PEG, which was further purified by ion-exchange chromatography. The product was converted to NHS-active ester using DCC and NHS to yield PEG-NHS, which was used for the conjugation of amino groups on Hb, SOD, and CAT (Scheme 2.2). Before cross-linking the



Scheme 2.2. Representation of cross-linking Hb with SOD and CAT employing dicarboxymethylated poly(ethylene glycol) (2 kDa).

activated PEG diacid into NHS, it was characterized by ^{1}H NMR spectroscopy and thin-layer chromatography.

2.3.4 Hb conjugation with SOD and CAT

To produce Hb-only conjugations, isolated bovine Hb was conjugated with PEG by varying the Hb:PEG molar ratio between 1:5 and 1:30. To produce Hb conjugations with SOD and CAT, Hb:PEG ratios identical to those used in Hb-only conjugations were used, and the ratio between SOD and CAT was fixed between 30000 to 300000 enzyme units in all conjugation reactions (*24, 28*). All Hb conjugation reactions in the PBS buffer (pH 7.4 with and without SOD and CAT) were conducted by adding activated PEG and stirring for 3h at 4 °C. Unreacted PEG and protein components were removed using 100-kDa dialyzing membranes and changing the PBS buffer for 24h. The product was further concentrated to the desired concentration using Amicon centrifugation filters, filter sterilized using a 0.22-μm pore size syringe filter, and stored at 4 °C until further use.

2.3.5 SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of PEG-conjugated Hb with and without SOD and CAT was performed on 4% to 12% Bis-Tris NuPAGE gels under reducing conditions by employing the XCell SureLock Mini-Cell apparatus (Invitrogen Life Technologies, Carlsbad, CA). The

samples were heated (denatured) at 70 °C for 10min before 1-to-10-µg-equivalent Hb protein was (20µl volume) applied to the gel. Electrophoresis was conducted at a constant 150 V for approximately 90min. The SeeBlue Plus2® prestained molecular weight standard was added to all gels and separation achieved using the NuPAGE® MES SDS running buffer in accordance with the manufacturer's (Invitrogen's) instructions. In order to observe the products, the gels were rinsed with ultrapure water (Millipore) and stained by SimplyBlue^{TM} safe stain (Invitrogen), followed by extensive washing in pure water for protein detection. The completion of the conjugation process was assessed by comparing it with appropriate molecular weight standards.

2.3.6 Size exclusion chromatography analysis of conjugates

Size exclusion chromatography (SEC) was performed employing the Agilent 1100 Liquid chromatography separation system for determining the weight-average molecular weight with absorbance detection at 280 nm (Agilent Technologies, Santa Clara, CA). The separation was performed on a BioSEP-S-4000 column (hydrophilic-bonded silica of 5-µm particle size and 500 A^{o} pore size) capable of resolving high-molecular- weight proteins ranging in size from 15 kDa to 2000 kDa (Phenomenex,Torrance, CA). The column was equilibrated using the mobile phase (0.1 M potassium phosphate buffer, pH 7.4).Samples were diluted in PBS and separated through the column at a flow rate of 0.5 ml/min at room temperature. Fifty microliters of cross-linked Hb-Hb and Hb-SOD-CAT conjugate solutions were injected, and the molecular weight of the samples

determined from a standard curve derived from the retention times of protein reference standards using the Sigma molecular mass marker. Molecular standards include carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), beta-amylase (200 kDa), apoferritin (443 kDa), thyroglobulin (669 kDa), and Dextran Blue (2000 kDa). All the data were analyzed using Agilent Chemstation software. The molecular weights and their distributions by SEC were represented and described in accordance with Hu Tao (*29*) and Nonier MF (*30*).

2.3.7 Analysis of absorption spectra of conjugated Hb

Employing a UV-visible SpectraMax spectrophotometer (CA, USA), the qualitative analysis of conjugated Hb was conducted by measuring the visible absorption spectrum of conjugated Hb with and without SOD and CAT. Twenty-µM Hb conjugates was placed in a UV cuvette and the visible absorption spectrum was scanned between 450 and 750 nm for 2 seconds.

2.3.8 Methemoglobin quantification

The quantity of methemoglobin in conjugated samples was determined using a method described by Patton et al. (*31*). Briefly, a sufficient quantity of the conjugated protein was placed in a UV-visible cuvette and diluted such that it provided an absorbance of less than one unit at 630 nm, referred to as A1. This value corresponded to the total Hb content, which consisted of oxy-, deoxy-, and methemoglobin content, in the sample. The sample was then treated with one

drop (50 µL) of potassium cyanide solution (1 part 10% KCN and 1 part PBS pH 7.5) and allowed to react for 5 to 10min. A second absorbance measurement referred to as A2 was performed on the same sample at 630 nm. The difference between A2 and A1 was calculated, and the methemoglobin content in the samples was calculated using the extinction coefficient value for methemoglobin $(3.7\ \text{cm mM})^{-1}$. The total Hb content in the sample was measured by placing the same quantity of conjugates in another cuvette and adding one drop (50 µL) of 20% $K_3Fe(CN)_6$ with a dilution. After the conversion of the oxy- and deoxy-Hb in the sample into methemoglobin, another drop of 10% KCN was added and the absorbance peak at 540 nm was measured (A3). Using the extinction coefficient of 11 $(\text{cm mM})^{-1}$ for cyanomethemoglobin, the Hb content was calculated using the equation below, and the percentage of methemoglobin was calculated from the ratio of total methemoglobin to Hb content in the sample.

$$\text{Methemoglobin} = \frac{\text{A1 - A2}}{3.7} \times \text{dilution factor}$$

$$\text{Hemoglobin} = \frac{\text{A3}}{11} \times \text{dilutionfactor}$$

$$\text{Methemoglobin \%} = \frac{\text{Methemoglobin}}{\text{Hemoglobin}} \times 100$$

2.3.9 SOD enzymatic assay

The level of SOD activity was determined using a previously reported cytochrome C reduction assay method (*32-34*). Specifically, SOD activity was measured by determining the rate of reduction of cytochrome C inhibition by the

superoxide radical generated using a xanthine and xanthine oxidase system at pH 7.8 and room temperature. The samples contained a mixture of 50 mM of potassium phosphate, 0.01 of mM cytochrome C, 0.1 mM of ethylenediaminetetraacetic acid, 0.05 mM of xanthine, 0.005 units of xanthine oxidase, and conjugated Hb. The uninhibited rate of reduction was adjusted by changing the quantity of the xanthine oxidase enzyme used in the assay such that the reaction produced an initial change in the absorbance between 0.025 - 0.005 per min at 550 nm. A 50% rate reduction of cytochrome C in the inhibited samples was considered to correspond to one unit of SOD. The background activity by the Hb component in the antioxidant-conjugated samples was subtracted by assaying equivalent activity in Hb-only conjugated samples. The inhibition rate and number of enzyme units per mL were calculated using the equations below.

$$\text{Percent inhibition} = \frac{\text{Change in absorbance per min at 550nm in uninhibited sample}}{\text{Change in absorbance per min at 550nm in uninhibited - blank sample}} \times 100$$

$$\frac{\text{Units}}{\text{mL}} \text{ of enzyme} = \frac{\text{percent inhibition} \times \text{dilution factor}}{(50\%) \times \text{volume of enzyme sample used in mL}}$$

2.3.10 CAT enzymatic assay

CAT activity was measured by monitoring the decomposition rate of H_2O_2 at 240 nm (*35-37*). Specifically, CAT was assayed in the conjugates by measuring the enzyme-catalyzed decomposition rate of H_2O_2 in accordance with the Sigma-Aldrich test procedure for the enzymatic assay of CAT (Sigma Aldrich, EC 1.11.1.6). A fresh 0.036% (w/w) H_2O_2 solution was prepared in 50 mM of potassium phosphate buffer at pH 7.0 and room temperature such that its initial absorbance was 0.550 units. 2.9 ml of this reagent was mixed with 0.1 ml of conjugated proteins in a cuvette, and the time required for a reduction in absorbance from 0.45 to 0.40 absorbance units was calculated. The number of enzyme units in the sample was calculated as per the equation below.

$$\text{Enzmye } \frac{\text{Units}}{\text{mL}} =$$

$$\frac{(3.45) \times \text{Dilution Factor}}{\text{Time in minutes for decrease in absorbance from } 0.45 \text{ to } 0.40 \times (0.1\text{ml of enzyme sample})}$$

The value of 3.45 in the equation refers to the decomposition of 3.45 μM of H_2O_2 in a 3.0-ml reaction mixture that decreases the absorbance from 0.45 to 0.40 absorbance units. The background activity of Hb in the samples was

removed by calculating the equivalent Hb activity in conjugated samples without SOD and CAT.

2.3.11 Combined peroxide and superoxide challenge

To examine the protective effect of Hb in Hb-antioxidant enzyme conjugates, the 1:10 Hb-Hb and Hb-SOD-CAT conjugates were tested by challenging them with superoxide anions generated by a xanthine (1mM)/xanthine oxidase (10 and 20 mUnits/mL) system and H_2O_2 (0.1 mM and 1 mM), both of which generate hydroxyl free radicals. Twenty μM of Hb equivalents were used in every reaction that had been incubated with free radicals for 30 min, 3 h, 24 h, and 48 h at room temperature in UV-visible cuvettes. The protective effect of Hb and its conversion to non-O_2-binding methemoglobin was evaluated using UV-visible spectrophotometry.

2.3.12 Statistical analysis

The statistical significance of the difference between conjugated Hb with and without SOD and CAT was analyzed by performing one-way analysis of variance (ANOVA) testing followed by Holm-Sidak testing to compare group means for differences between enzymatic activity and polymer conjugation ratios and to determine the conjugation and storage effect on methemoglobin formation. For all analyses, a *p* value less than 0.05 was considered to indicate statistical significance.

2.4 Results

2.4.1 SDS-PAGE

The results of SDS-PAGE analyses indicated the formation of cross-linked products at all Hb:PEG ratios (1:5, 1:10, 1:15, 1:20, and 1:30) both with and without SOD and CAT. A range of different molecular weight conjugates were obtained (~100 kDa products and >180 kDa) when compared to the molecular weight standards used in SDS-PAGE (Figure 2.1). The smear-like or ladder-like appearance of the SDS-PAGE results indicates the differential mobility of the conjugates due to differences in the sizes/molecular weights of the polymerized conjugates. However, SDS analysis demonstrated only the formation of the polymerized products, and did not specify the exact size of the conjugates with molecular weights above the detection limit of 180 kDa. The results of these analyses provided the first piece of confirming evidence regarding the cross-linking of Hb-Hb and Hb-SOD-CAT conjugates.

2.4.2 SEC analysis of conjugates

SEC analysis confirmed the formation of large PEG cross-linked Hb conjugates both with and without SOD and CAT with a broad elution spread in all Hb:PEG molar ratios (Figure 2.2). When Hb-only conjugates were polymerized employing PEG, a heterogeneous population of cross-linked Hb was obtained with molecular weights ranging from ~150 kDa to ~2000 kDa. The chromatogram shows more prominence toward the lower retention times (the left side) than the higher retention times (the right side) of the broad peak, indicating that the

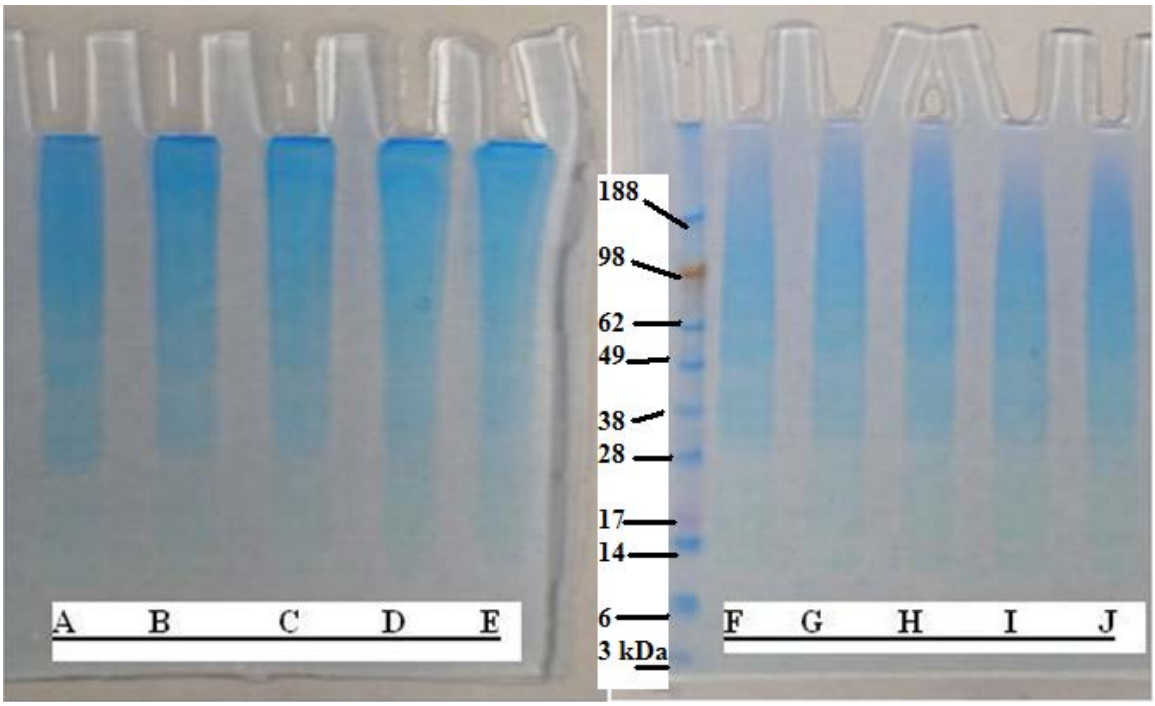


Figure 2.1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis showing formation of Hb cross-linked products with and without SOD and CAT (Hb:PEG). Lane A, 1:5 Hb-Hb; Lane B, 1:10 Hb-Hb; Lane C, 1:15 Hb-Hb; Lane D, 1:20 Hb-Hb; Lane E, 1:30 Hb-Hb; Molecular Weight Standard; Lane F, 1:5 Hb-SOD-CAT; Lane G, 1:10 Hb-SOD-CAT; Lane H, 1:15 Hb-SOD-CAT; Lane I, 1:20 Hb-SOD-CAT; Lane J, 1:30 Hb-SOD-CAT.

majority of the cross-linked Hb-only conjugates were comprised of a high molecular weight species of ~2000 kDa. Different Hb/PEG ratios yielded similar molecular weight distribution profiles. When SOD and CAT were added during cross-linking, the chromatograms of the conjugates show a molecular weight ranging from ~100 kDa to ~1500 kDa. However, chromatograms of Hb conjugates with SOD and CAT show a major peak shift toward higher retention times of ~100 kDa time (the right side), unlike Hb-only conjugates. Minimal differences were observed among conjugates of different Hb:PEG ratios,

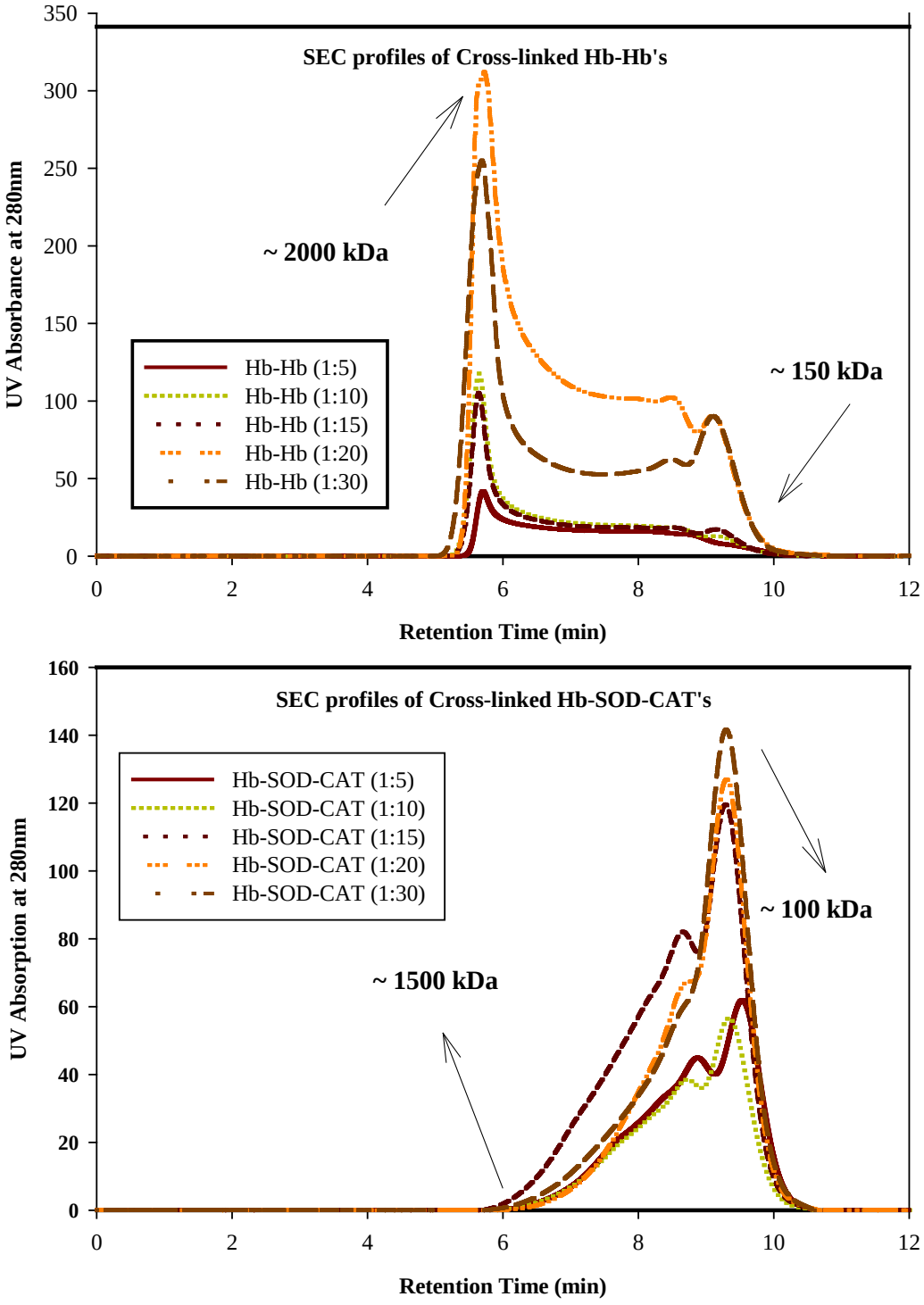


Figure 2.2. SEC of cross-linked Hb with and without SOD and CAT of Hb-PEG ratios from 1:5 to 1:30 and a SOD-CAT enzyme ratio of 30000:300000 units.

including among those conjugates with SOD and CAT. The average molecular weight for all the conjugates appeared to range from ~800 to 1000 kDa, with broad peak profiles and dramatic differences in elution profiles for the conjugation of Hb with and without SOD and CAT.

2.4.3 Visible absorbance spectra of conjugated Hb

Oxyhemoglobin has two characteristic absorbance peaks at 540 and 575 nm and appears red in the solution state, whereas methemoglobin has one characteristic peak at 630 nm and appears dark brown. As shown in Figure 2.3, when Hb was self-cross-linked, an increased quantity of methemoglobin formation resulted with increasing amounts of the cross-linking agent PEG. The change in absorbance was found to vary from 0.01 to 0.2 units when Hb was self-cross-linked at Hb:PEG molar ratios between 1:5 and 1:30. A significant change in the absorbance at 630 nm was observed at Hb:PEG cross-linking ratios of 1:15, 1:20, and 1:30. However, the addition of SOD and CAT during cross-linking significantly decreased the formation of methemoglobin, as demonstrated by the lower absorbance at 630 nm. This decrease is clearly significant up to a 1:20 Hb:PEG ratio with SOD and CAT, with only a slight increase observed at a 1:30 Hb:PEG ratio. The change in absorbance varied between 0.01 and 0.06 units, dramatically less than that of self-cross-linked Hb.

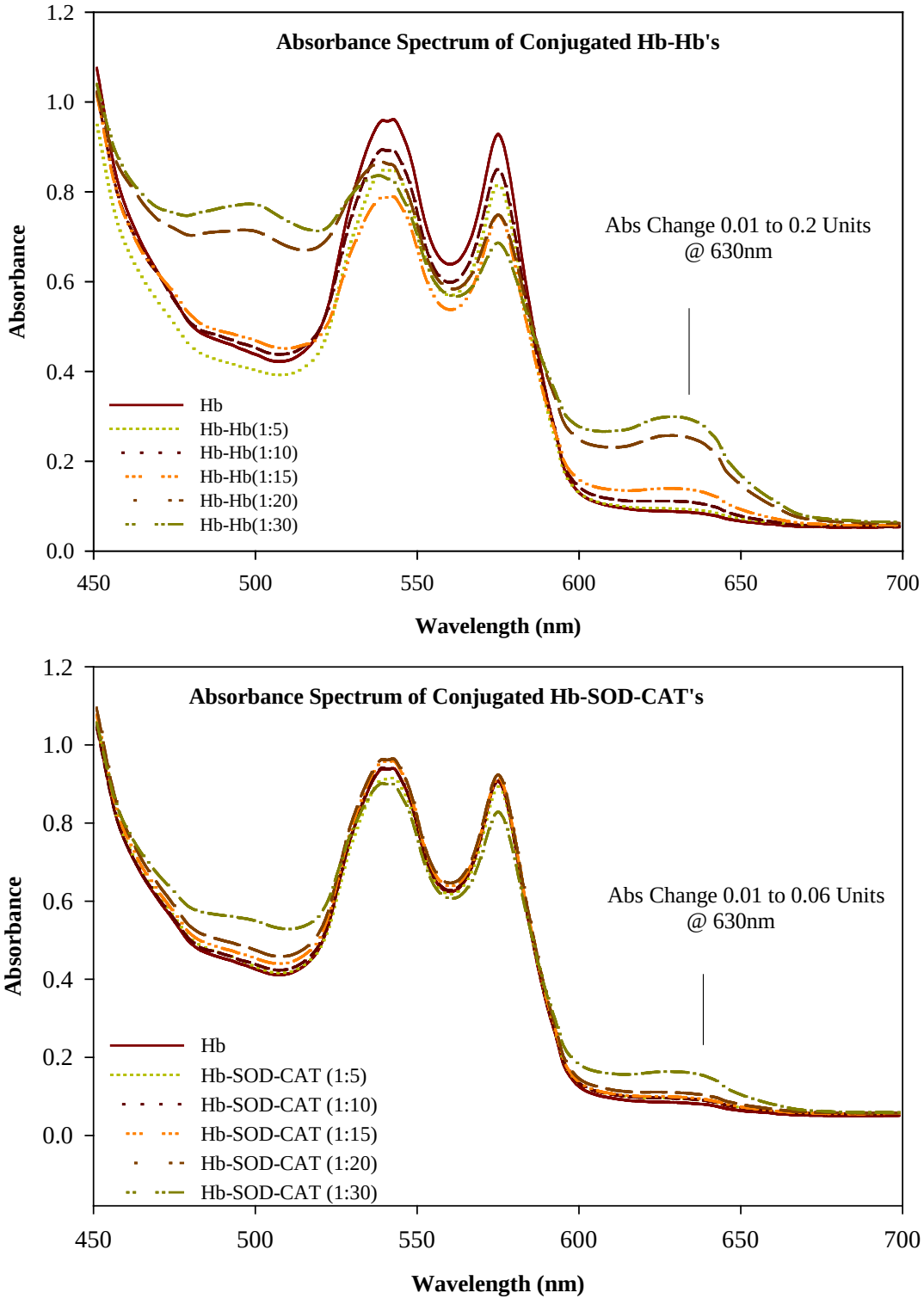


Figure 2.3. Absorbance spectra of cross-linked Hb with SOD and CAT 24h after cross-linking, Hb:PEG ratios 1:5 to 1:30 and SOD/CAT enzyme ratio of 30000:300000 units.

2.4.4 Methemoglobin quantification

As shown in Figure 2.4, the cross-linking of Hb by PEG enhanced the conversion of Hb to methemoglobin in the absence of antioxidant enzymes. For self-cross-linked Hb, the methemoglobin content increased within one day when an increasing quantity of PEG-conjugating agent was added. This increase was greater than 10% for an Hb:PEG ratio of 1:15 and greater than 40% for ratios of 1:20 and 1:30. Such Hb-only conjugates are unlikely to be useful as O_2 carriers due to the large quantity of methemoglobin unless a mechanism to reverse this process can be incorporated. One such mechanism may be the addition of antioxidant enzymes, which in this study led to a significant decrease in the quantity of methemoglobin at all Hb:PEG ratios during cross-linking. The complete absence of methemoglobin in Hb conjugates with SOD and CAT was observed at Hb:PEG ratios of 1:5, 1:10 and 1:15, and only 1.6 ± 0.6% was observed at the 1:20 ratio and 7.3 ± 1.3% at the 1:30 ratio. These results indicate that the conjugating agent had a significant effect on the methemoglobin content, emphasizing the need for the addition of antioxidant enzymes to inhibit the conversion of Hb to methemoglobin. Although methemoglobin is formed in all cross-linking without antioxidants, the results of statistical analysis indicated significant differences between Hb-only and Hb-SOD-CAT cross-linked formulations only at Hb:PEG ratios of 1:15, 1:20, and 1:30 (p-value < 0.001).

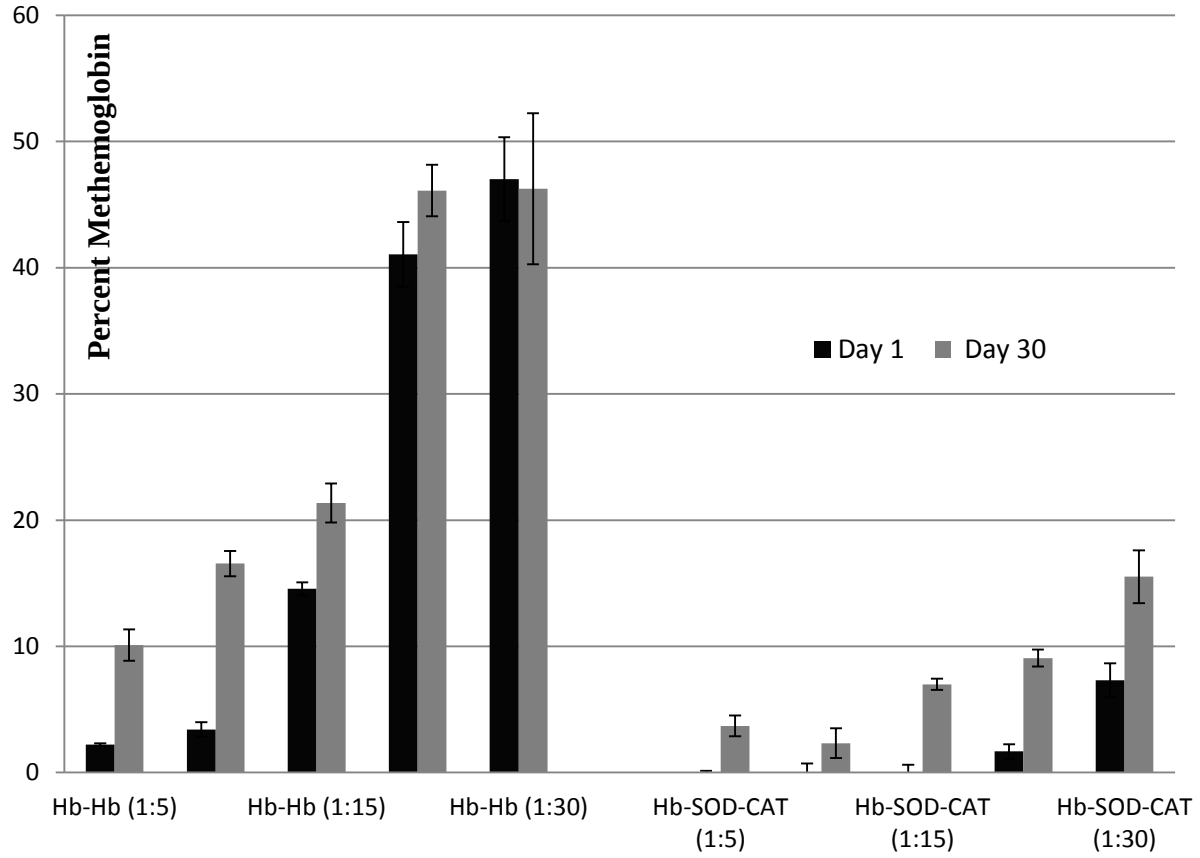


Figure 2.4. Effect of SOD and CAT on methemoglobin formation in cross-linked Hb during storage at 4 ^{0}C for one month, with Hb:PEG ratios 1:5 to 1:30 and a SOD/CAT enzyme ratio of 30000:300000 units.

The results also indicate that self-cross-linked Hb conjugates stored at 4 ^{0}C for one month will develop methemoglobin content of more than 10%. A significant increase of methemoglobin was observed at Hb:PEG ratios of 1:5 (from 2.2 ± 0.1% to 10 ± 1.2%), 1:10 (3.4 ± 0.6% to 16.6 ± 1%), and 1:15 (14.6 ± 0.5% to 21.4 ± 1.5%). The results of statistical analysis also indicated a statistically significant increase in the quantity of methemoglobin among all

Hb/PEG ratios (1:5 to 1:30) of cross-linked Hb at days 1 and 30 without SOD and CAT (p-value < 0.001). However, for cross-linked Hb with SOD and CAT, the percentage of increase in methemoglobin content was less than 10% in Hb/PEG ratios of 1:5, 1:10, 1:15, and 1:20, with only the ratio of 1:30 showing an increased methemoglobin content (from 7.3 ± 1.3% to 15.52 ± 2.1%). These results provide evidence for the beneficial effect of the addition of SOD and CAT for the long-term protection of Hb against oxidative stresses during storage.

2.4.5 Retention of SOD enzymatic activity

For the effective removal of superoxide anion free radicals and the protection of Hb from free radicals, the enzymatic activity of conjugated SOD must be preserved after cross-linking with PEG and Hb in the final conjugate mix. The results show that the enzymatic activity of the conjugated SOD was reduced in comparison to the enzymatic activity of the control (free) SOD (Table 2.1). However, the enzymatic activity of SOD in all the conjugates was shown to be 70 to 80% of the original. Although the enzymatic activity decreased compared to the enzymatic activity of the control SOD, there was no statistically significant differences among the conjugated Hb with SOD at different PEG ratios (p = 0.154). This indicates that increasing the quantity of the PEG used in conjugation reactions has no significant effect on SOD enzymatic activity.

Table 2.1. Percentage retention of SOD enzymatic activity in Hb conjugates after cross-linking with modified PEG

Conjugate (Hb:PEG)	**SOD enzymatic activity** [a)] **Percentage retained** [b)]
Hb-SOD-CAT (1:5)	73 ± 2
Hb-SOD-CAT (1:10)	79 ± 3
Hb-SOD-CAT (1:15)	76 ± 6
Hb-SOD-CAT (1:20)	72 ± 5
Hb-SOD-CAT (1:30)	67 ± 5

[a)] Based on the enzymatic activity of free SOD added before conjugation; [b] Values are means ± SD (*n* =3).

2.4.6 Retention of CAT enzymatic activity

For the effective removal of peroxide free radicals in the protection of Hb from free radicals, the enzymatic activity of conjugated CAT must be preserved after cross-linking with PEG in the final conjugate mix. As shown in Table 2.2, the enzymatic activity of conjugated CAT is similar to the enzymatic activity of free CAT (the control). The CAT activity of all the conjugates was estimated to be between 90 and 99%. Increasing the quantity of PEG polymers for conjugating Hb with CAT resulted in no statistically significant differences in enzymatic activity (p = 0.382).

Table 2.2. Percentage retention of CAT enzymatic activity in Hb conjugates after cross-linking with modified PEG

Conjugate (Hb:PEG)	**CAT enzymatic activity** [a)] **Percentage retained** [b)]
Hb-SOD-CAT (1:5)	91 ± 6
Hb-SOD-CAT (1:10)	93 ± 2
Hb-SOD-CAT (1:15)	96 ± 3
Hb-SOD-CAT (1:20)	91 ± 4
Hb-SOD-CAT (1:30)	90 ± 4

[a)] Based on the enzymatic activity of free CAT added before conjugation; [b] Values are means ± SD (*n* =3).

2.4.7 Protection of Hb from H_2O_2

Challenging Hb-only conjugates (Hb/PEG 1:10 molar ratio, 20 μM) and Hb-SOD-CAT conjugates with H_2O_2 (0.1mM and 1mM) for 0.5, 3, 24, and 48h led to identification of a significant protective effect on cross-linked Hb with SOD and CAT, as shown in Figures 2.5 and 2.6. When Hb-only cross-linked conjugates were challenged with 1 mM of H_2O_2, the absorption peak for methemoglobin appeared within 30min of incubation, with the increase in absorbance at 630 nm being approximately 0.11 units at 30min and 0.15 units at 48h. The corresponding characteristic Hb absorbance peak decrease was found to be 0.33 (30min) and 0.46 (48h) units at 540 nm, and 0.37 (30min) and

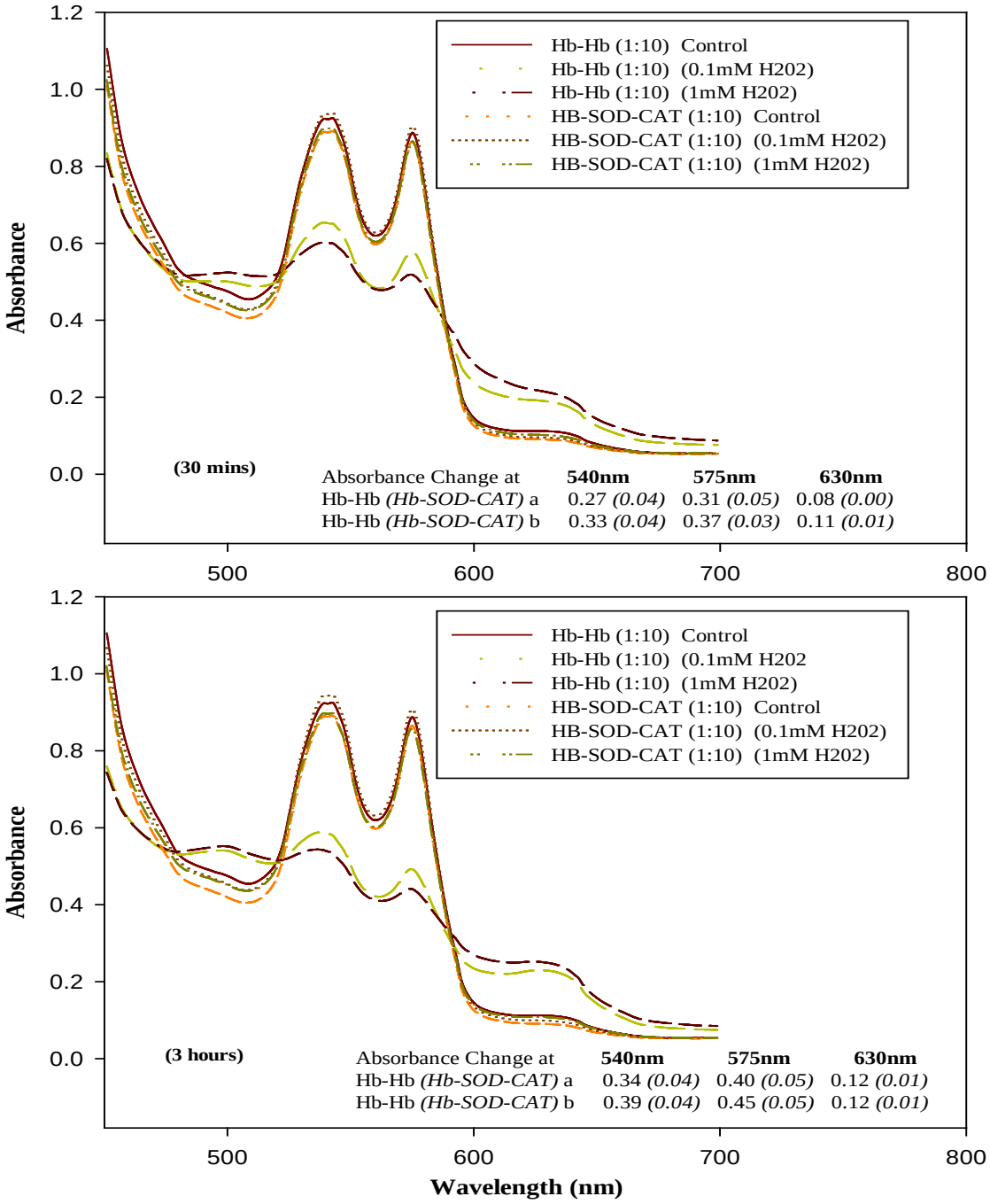


Figure 2.5. Protective effect of SOD and CAT on cross-linked Hb (Hb:PEG ratios of 1:5 to 1:30 and SOD/CAT enzyme ratio of 30000:300000) challenged with 0.1 and 1 mM H_2O_2 for 30min and 3h. Change in absorbance values represents difference between free Hb and cross-linked products. Values in parentheses refer to conjugates with SOD and CAT: a) refers to conjugates challenged with 0.1 mM of H_2O_2 and b to conjugates challenged with 1mM of H_2O_2.

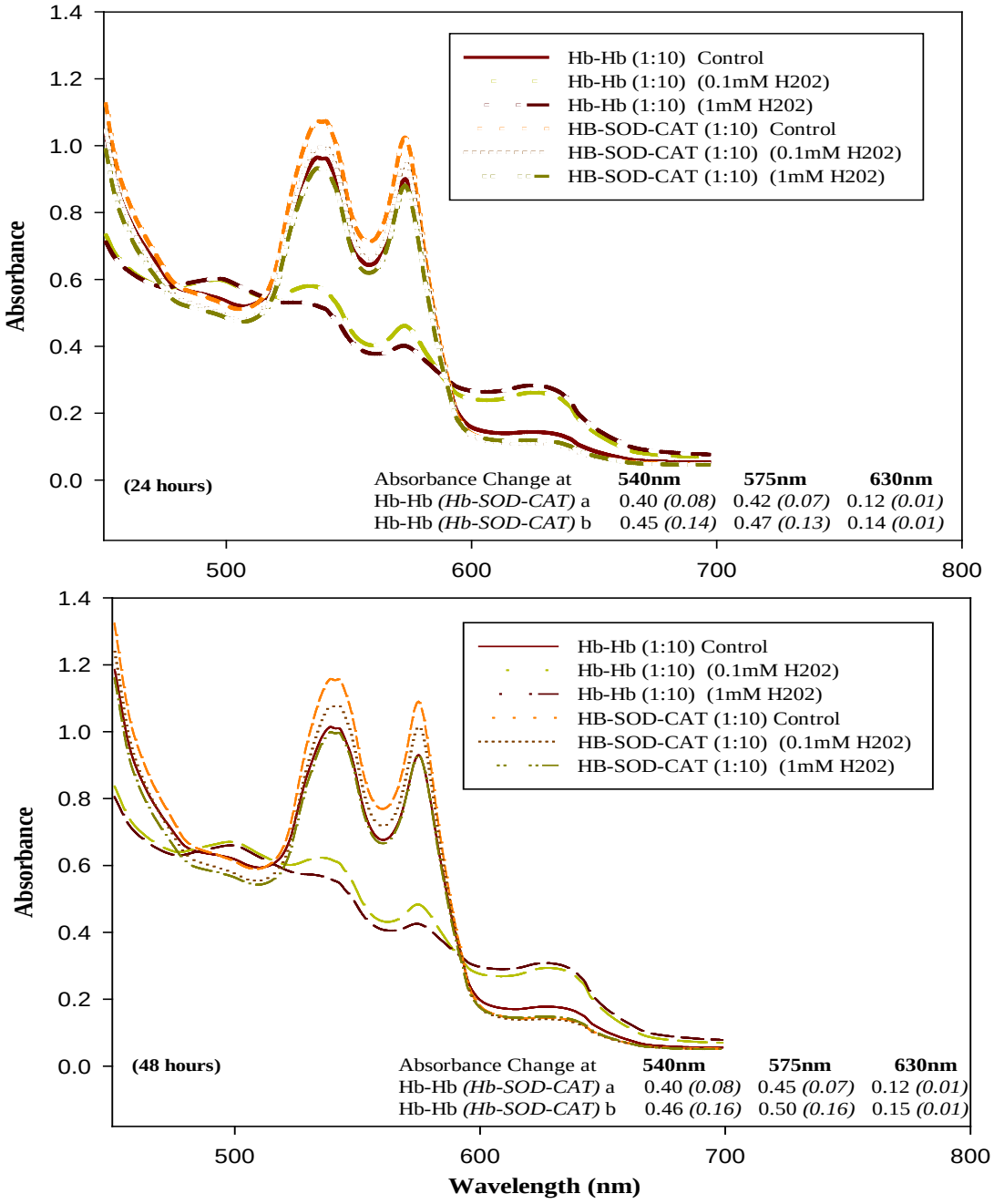


Figure 2.6. Protective effect of SOD and CAT on cross-linked Hb (Hb:PEG ratios of 1:5 to 1:30 and SOD/CAT enzyme ratio of 30000:300000) challenged with 0.1 and 1mM of H_2O_2 for 24 and 48h. The change in absorbance value represents the difference between free Hb and cross-linked products. Values in parentheses refer to conjugates with SOD and CAT: a) refers to conjugates challenged with 0.1 mM of H_2O_2 and b to conjugates challenged with 1mM of H_2O_2.

0.50 (48h) units at 575 nm. The results further explain the similar trend of Hb-only conjugates showing damage/degradation at both concentrations (0.1 and 1mM) of H_2O_2 used in the *in vitro* experiments. The insignificant increase in the methemoglobin content peak after 24h in Hb-only formulations may have been due to the complete degradation of Hb or further degradation of Hb into ferryl Hb or other Hb degradation products (*38*).

The changes in the characteristic Hb absorbance peak at 540 and 575 nm in Hb-only conjugates indicate significant Hb damage and near complete degradation of Hb at 48h. The results also indicate that a small quantity of peroxide is sufficient to cause Hb damage and render it unsuitable as an O_2 carrier, demonstrating that even though cross-linking may stabilize the tetramers of isolated Hb, free radicals can easily damage the unprotected Hb. On conjugating Hb with SOD and CAT, the cross-linked formulation demonstrated significant Hb-protective effects. The results also show that the increase in the absorbance of methemoglobin peaks were less than 0.01 units in all cases at 48h of challenge at room temperature, indicating near complete Hb protection from H_2O_2-generated free radical**s.** The graphical overlay of Hb not challenged by H_2O_2 was very similar to that of Hb conjugates containing SOD and CAT that were challenged by H_2O_2. These results strongly indicate that the incorporation of SOD and CAT may provide Hb with enhanced protection against the damaging effects of hydroxyl and peroxide radicals.

2.4.8 Protection of Hb from superoxide

Challenging (Hb:PEG 1:10 molar ratio) conjugates with xanthine (1 mM) and a xanthine oxidase mixture (10 mU/mL and 20 mU/mL) demonstrated inhibition of Hb conversion to methemoglobin, with inhibition of decreased Hb absorption peaking at 540 and 575 nm, only if SOD and CAT were included in the conjugation reaction (Figures 2.7 and 2.8). When Hb-only cross-linked conjugates were challenged, a slightly increased absorbance at 630 nm was observed and a significant decrease in absorption at 540 and 575 nm, indicating a reduction in the Hb content within 30min and a continual decrease over a period of 3h. However, Hb conjugates with SOD and CAT demonstrated absorption profiles very similar to those of unchallenged conjugates, indicating near complete prevention of Hb degradation and inhibition of free-radical damage from the superoxide anion for at least 3h (Figure 2.7).

Compared to unchallenged Hb, the absorption spectra of the conjugates without SOD and CAT after 24 h of superoxide free-radical challenge (Figure 2.8) show a significant decrease in absorbance at 540 nm (0.10 units with 10 mU/mL xanthine oxidase and 0.16 units with 20 mU/mL xanthine oxidase) and at 575 nm (0.16 units with 10 mU/mL xanthine oxidase and 0.24 units with 20 mU/mL xanthine oxidase). They also show a significant increase in methemoglobin absorbance at 630 nm (0.13 units with 10 mU/mL xanthine oxidase and 0.15 units with 20 mU/mL xanthine oxidase) compared to unchallenged conjugated Hb. Although there was a considerable increase

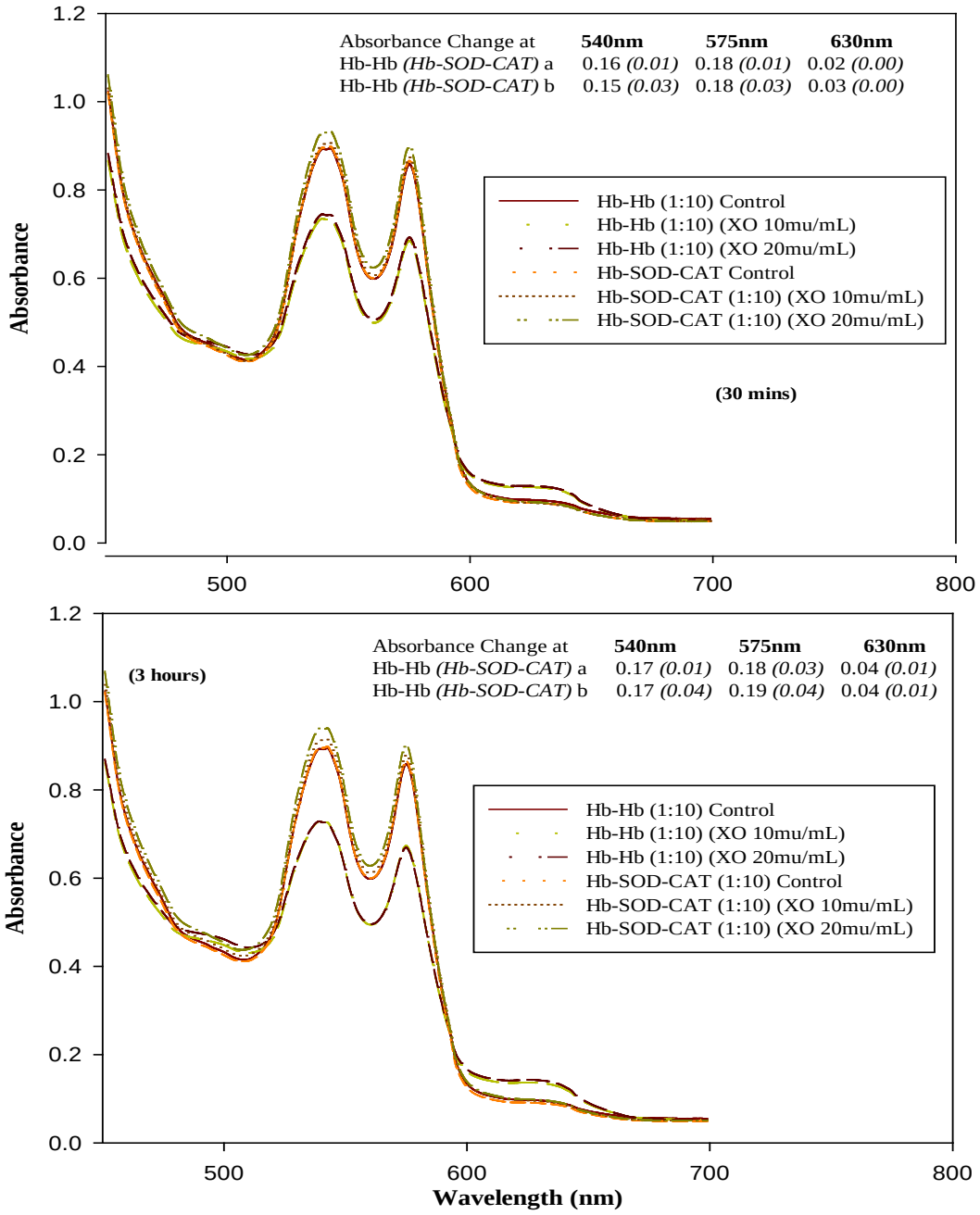


Figure 2. 7. Protective effect of CAT and SOD on cross-linked Hb (Hb:PEG ratios of 1:5 to 1:30 and SOD:CAT enzyme ratio of 30000:300000) when challenged with 1 mM xanthine and 10 and 20 mUnits/mL xanthine oxidase for 30min and 3h. Change in absorbance values refers to differences between free Hb and cross-linked products. Values in parentheses refer to conjugates with CAT and SOD: a) refers to conjugates challenged with 1 mM xanthine and 10 mUnits/mL xanthine oxidase and b to conjugates challenged with 1mM xanthine and 20 mUnits/mL xanthine oxidase.

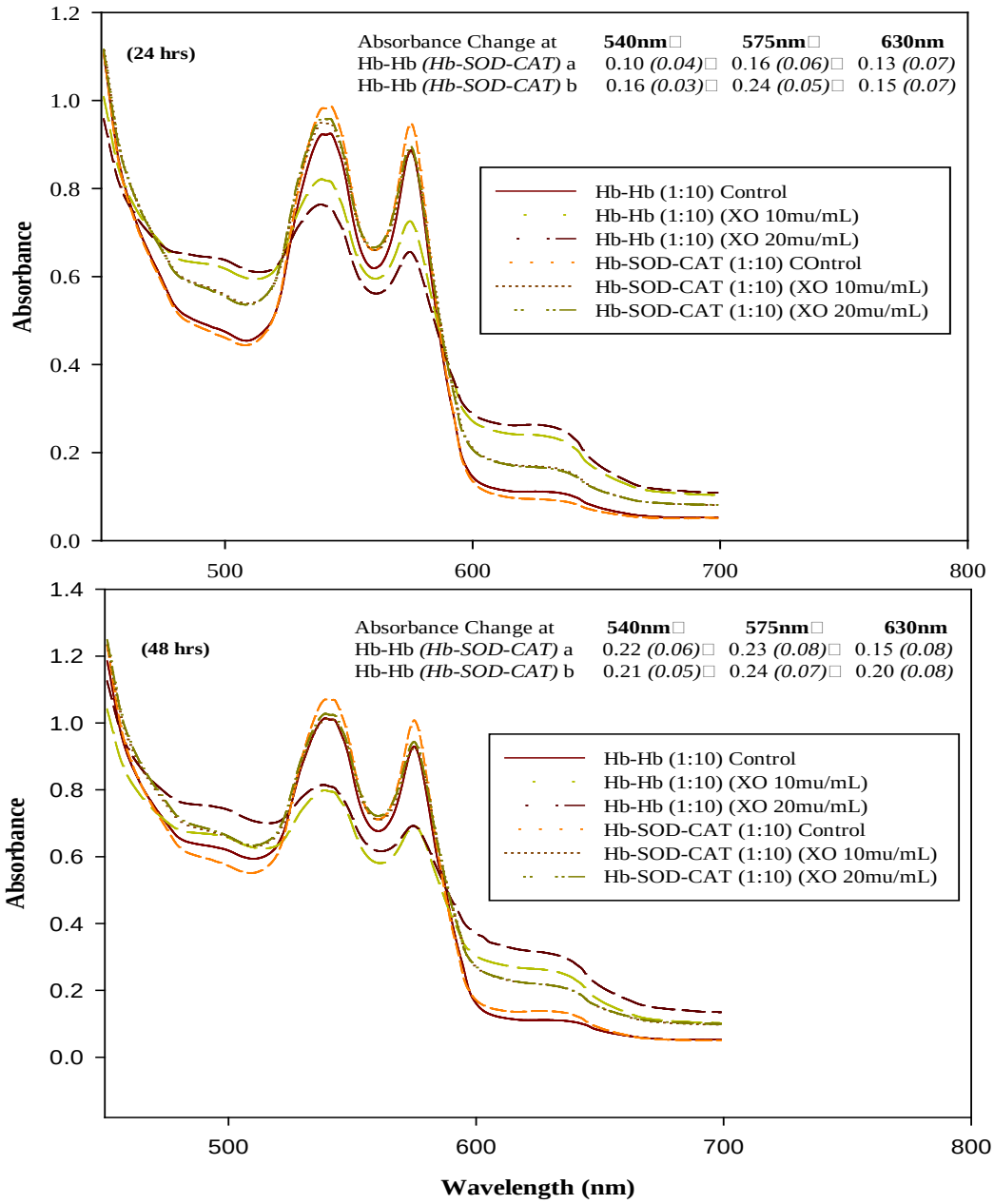


Figure 2.8. Protective effect of CAT and SOD on cross-linked Hb (Hb:PEG ratios of 1:5 to 1:30 and SOD:CAT enzyme ratio of 30000:300000) challenged with 1 mM xanthine and 10 and 20 mUnits/mL xanthine oxidase for 24 and 48h. Change in absorbance values refers to differences between free Hb and cross-linked products. Values in parentheses refer to conjugates with CAT and SOD: a) refers to conjugates challenged with 1 mM xanthine and 10 mUnits/mL xanthine oxidase and b to conjugates challenged with 1mM xanthine and 20 mUnits/mL xanthine oxidase.

(from 0 to 0.07 units) in the quantity of methemoglobin in formulations with SOD and CAT, the increase was less than that of formulations without SOD and CAT (0.07 with and 0.15 units without SOD and CAT), emphasizing the importance of antioxidant enzymes in the conjugates. A similar trend is revealed in the absorption spectra of the conjugates after 48h, indicating that Hb conjugates with antioxidants are afforded better protection than Hb-only conjugates at all time periods. Although the results indicate that complete protection from the superoxide anion may be achieved by increasing and further optimizing the quantity of SOD enzyme in the conjugates, it should be noted that Hb was challenged with larger amounts of superoxide anions than may be expected *in vivo*.

2.5 Discussion

The SDS-PAGE distribution of the Hb-only conjugate clearly shows a heterogeneous distribution of conjugates in the cross-linked formulation differing little from the Hb polymerized with SOD and CAT. The band distributions of SOD and CAT in the gel overlapped with those of Hb, a type of SDS-PAGE band pattern often observed and expected in PEG-driven chemistries in which chemical modifications are or cannot be tightly controlled (*39*). This type of pattern indicates a high degree of intramolecular cross-linking within the Hb molecule to stabilize the tetrameric structure, as well as a high degree of intermolecular cross-linking among Hb, SOD, and CAT, resulting in an overall increase in the molecular weight of the Hb conjugates. However, the

average molecular weight for conjugates of different PEG and Hb ratios only slightly differed from those with similar distribution profiles. For Hb-SOD-CAT conjugates, the shift in their intensity peak toward lower-molecular-weight conjugates or their preference for formation of lower molecular conjugates may indicate a decrease in intermolecular cross-linking. Despite changes in their elution profiles, as had been expected, it appears that heterogeneous mixtures of Hb conjugates are suitable for functions such as encapsulation with isolated cells due to their ability to provide O_2 to isolated encapsulated cells (*13*).

The polymerization method involves conjugation of amine groups on amino acid residues, primarily lysine, within the Hb and on the antioxidant enzymes (*24*). A recent study demonstrated that the conjugation of PEG chains to Hb lysine residues by thiolation-mediated PEGylation induced structural changes by increasing the hydration shell, which assisted in the stabilization of a relaxed, high-affinity conformation (*40*). The study found that methemoglobin levels must be maintained below 10% in PEG-conjugated Hb for the efficient oxygenation of tissues (*41*) and that methemoglobin levels should remain below (< 10%) for extended periods following PEG-Hb administration (*41*).

Other research has found that human Hb conjugated to maleimide-activated poly(ethylene glycol) increases *in vitro* auto-oxidation at room temperature and ROS receptivity to enhanced oxidation. Specifically, the rate of *in vitro* heme loss was found to increase fivefold once conjugates were oxidized to methemoglobin, which was attributed to the loss of heme in both alpha and beta chains (*42*). PEGylation of Hb at Cys-93 (beta) and Val-1 (beta) was shown

to perturb the environment in the heme pocket and increase auto-oxidation, an effect shown to be the direct effect of the chemistry of conjugation and not of the PEG molecules. However, the PEG chains enhanced auto-oxidation by promoting the nucleophilic attack of heme in the presence of H_2O_2 by increasing the quantity of water molecules in the Hb hydration layer (*23*). Even though the exact mechanism by which the quantity of methemoglobin increased in this study remains unknown, the results clearly demonstrated that the mechanism that increases methemoglobin formation during conjugation is significantly minimized or reduced by the addition of antioxidant enzymes, and may therefore be a selective chemical or physical Hb modification that provides efficient protection against oxidant stress during conjugation and storage at 4 ^{0}C. A recent study found that Hb-SOD chimeric protein conjugates co-expressed by the human alpha and beta Hb chain and manganese SOD gene together in *Escherichia coli* exhibited an auto-oxidation rate 44% lower than that of a control, and were thus beneficial (*43*).

In the conjugation method used in this study, PEG was conjugated to SOD lysine amino residues, primarily by acylation reaction, to yield amide bonding. Due to the large number of amines available on the SOD surface (20 in the dimeric enzyme)(*44*); heterogeneous products are generally obtained due to steric hindrance, differences in reactivity, and nucleophilicity. It has been shown that the loss of enzymatic activity of PEGylated SOD is related to a decrease in the ability to direct the O_2^- ion toward the active enzyme site (*45*). Some loss of enzymatic activity in the conjugation performed in this study may have been due

to the masking of positive charges by PEG involved in channeling the superoxide ion on the active site. It may also have been due to modification of the involvement of the positively-charged lysine amino group in the electrostatic loop that drives the substrate superoxide anion to the active site. The fact that an increasing quantity of PEG in the conjugation reaction did not further decrease the enzyme activity, as all 20 lysines in dimeric SOD enzyme are not involved in conjugation, may have been due to steric hindrance. The results obtained in this study are thus consistent with observations made by Veronese et al. (*44*), and indicate that the level of enzymatic activity depends on the type of conjugating agent used.

Catalase has approximately 112 amino groups (-NH2) available for chemical modification by the method of PEG cross-linking used in this study, but it is unclear how many are readily accessible for reaction. It was previously shown that only about 26 amino residue groups that were modified in a similar PEG-modified CAT process retained greater than 90% of their enzymatic activity. The slight decrease was attributed to the modification of the tertiary structure in the catalytic site by amidination of the amino group (*46*).

It has been shown that methemoglobin can be reduced in Hb-loaded nanoparticles by prereduction of raw Hb using sodium dithionite post-encapsulated with SOD and CAT (*9*). However, long-term stability, enzyme leaching, and the presence of Hb in a non-cross-linked or polymerized state may become concerns when using this approach. In normal *in vivo* conditions, Hb is present in ferrous form (Fe^{+2}), in which it functions as an effective O_2 carrier

protected by both an enzymatic and non-enzymatic-rich antioxidant environment. Methemoglobin, is converted back to Hb by the methemoglobin reductase (metHb) system and maintained in the Fe^{+2} state at levels above 98%. Spontaneous auto-oxidation of Hb causes the production of the superoxide anion ($O_2^{\cdot -}$) by removing the electron from the ferrous Hb and converting it into ferric (Fe^{+3}) methemoglobin. The superoxide anion causes increased H_2O_2 production by various indirect mechanisms and damage to Hb and its surrounding cellular environment (*47*). Under normal conditions, iron released from Hb or free Hb will be removed by complexation with haptoglobin, and thus heme-related toxicity is avoided (*38*). However, in excessively oxidative situations, Hb is converted into methemoglobin (Fe^{+3}) and ferryl Hb (Fe^{+4}) and releases globulin and free iron. The free iron reacts with H_2O_2 to produce the hydroxyl radical (OH·) by Haber-Weiss and Fenton chemistry (*47*), which further enhances the release of heme, which has been shown to cause cellular damage in both humans and animal models by different pathways (*48*). Simoni et al. demonstrated that particularly modified Hb solutions increased lipid peroxidation, and thus the cellular damage mediated by free-radical processes (*49*).

Recent studies have found that PEG cross-linked Hb-SOD-CAT with an Hb/PEG ratio of 1:20 contains less than 2% methemoglobin, in contrast to the reported 7% value for the cross-linked product immediately after cross-linking with a Hb/glutaraldehye ratio of 1:17. The content of methemoglobin with a Hb/glutaraldehye ratio of 1:17 was found to increase by 10% within only 5 days, compared to less than 10% for a Hb/PEG ratio of 1:20 within 30 days when

incubated at 4 ^{0}C (*50*). These results clearly illustrate one advantage of using PEG rather than glutaraldehyde, and it is anticipated that the conjugates will perform even better in long-term studies. Another advantage of using PEG-based conjugates is greater effectiveness in severely hypoxic and low-pressure conditions where the conjugate has a p50 of 7 mm Hg (*51*), as compared to using 25 mm Hg of high-p50 glutaraldehyde (*52*).

This study used Hb conjugates with an Hb:PEG ratio of 1:10 to test for the existence of a protective effect against free radicals because these conjugations had been shown to decrease methemoglobin content during conjugation and storage for one month. The results of this study demonstrate that cross-linking Hb with antioxidant enzymes clearly minimizes or inhibits Hb oxidation by free radicals, and thus Hb conversion to methemoglobin. Cases in which isolated cells, such as beta or cardiac cells, must be transplanted or stored require the use of Hb conjugates that protect cells against both hypoxic and free-radical stressors. Extending the life of an O_2-carrying agent with antioxidant enzymes may provide protection for a longer duration during isolated cell transplantation by reducing intracellular and extracellular oxidative stress. As the peroxide and superoxide radicals used in this *in vitro* study were of concentrations significantly greater than those present in inflamed or hypoxic states, it is anticipated that these conjugates will demonstrate superior functionality in *in vivo* situations in which much lower localized H_2O_2 concentrations are examined (*53, 54*).

2.6 Conclusion

The goal of this work was to develop Hb conjugates of large molecular weight cross-linked with antioxidant enzymes using a biocompatible polymer, in this case PEG. The results confirm that the approach used in this study leads to the formation of large PEG cross-linked Hb-SOD-CAT conjugates that have the capacity to protect Hb from free radicals and prevent its conversion to methemoglobin during conjugation and storage. The results thus indicate the utility of this approach in the co-encapsulation of conjugates with isolated pancreatic beta cells for their long-term protection, and thus the extension of their *in vivo* functionality in the treatment of diabetes, due to the large size of the conjugates, which helps prevent leakage of the cross-linked conjugates when co-encapsulated with isolated cells, and to the ability to supply O_2, which is crucial for cell viability, without Hb degradation.

2.7 Acknowledgments

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2.8 References

(1) Villela, N. R., Cabrales, P., Tsai, A. G., and Intaglietta, M. (2009) Microcirculatory Effects of Changing Blood Hemoglobin Oxygen Affinity During Hemorrhagic Shock Resuscitation in an Experimental Model. *Shock 31*, 645-52.

(2) Cabrales, P., Tsai, A. G., and Intaglietta, M. (2008) Isovolemic exchange transfusion with increasing concentrations of low oxygen affinity hemoglobin solution limits oxygen delivery due to vasoconstriction. *Am J Physiol Heart Circ Physiol 295*, H2212-8.

(3) Isaka, M., Imamura, M., Sakuma, I., Shiiya, N., Ishizuka, T., Ogata, Y., and Yasuda, K. (2007) Experimental study of pegylated liposomal hemoglobin on norepinephrine release and reperfusion arrhythmias in isolated guinea pig hearts. *Ann Thorac Cardiovasc Surg 13*, 391-5.

(4) Spahn, D. R., and Kocian, R. (2005) Artificial O2 carriers: status in 2005. *Curr Pharm Des 11*, 4099-114.

(5) Winslow, R. M. (2008) Cell-free oxygen carriers: scientific foundations, clinical development, and new directions. *Biochim Biophys Acta 1784*, 1382-6.

(6) Boykins, R. A., Buehler, P. W., Jia, Y., Venable, R., and Alayash, A. I. (2005) O-raffinose crosslinked hemoglobin lacks site-specific chemistry in the central cavity: structural and functional consequences of beta93Cys modification. *Proteins 59*, 840-55.

(7) Dimino, M. L., and Palmer, A. F. (2007) High O2 affinity hemoglobin-based oxygen carriers synthesized via polymerization of hemoglobin with ring-opened 2-chloroethyl-beta-D-fructopyranoside and 1-o-octyl-beta-D-glucopyranoside. *Biotechnol Bioeng 97*, 462-72.

(8) Graves, P. E., Henderson, D. P., Horstman, M. J., Solomon, B. J., and Olson, J. S. (2008) Enhancing stability and expression of recombinant human hemoglobin in E. coli: Progress in the development of a recombinant HBOC source. *Biochim Biophys Acta 1784*, 1471-9.

(9) Zhang, X., Liu, C., Yuan, Y., Shan, X., Sheng, Y., and Xu, F. (2008) Reduction and suppression of methemoglobin loaded in the polymeric nanoparticles intended for blood substitutes. *J Biomed Mater Res B Appl Biomater 87*, 354-63.

(10) Sakai, H., Horinouchi, H., Tsuchida, E., and Kobayashi, K. (2009) Hemoglobin-Vesicles and Red Blood Cells as Carriers of Carbon Monoxide Prior to Oxygen for Resuscitation after Hemorrhagic Shock in a Rat Model. *Shock 31*, 507-14.

(11) Bobofchak, K. M., Tarasov, E., and Olsen, K. W. (2008) Effect of cross-linker length on the stability of hemoglobin. *Biochim Biophys Acta 1784*, 1410-4.

(12) Alayash, A. I., D'Agnillo, F., and Buehler, P. W. (2007) First-generation blood substitutes: what have we learned? Biochemical and physiological perspectives. *Expert Opin Biol Ther 7*, 665-75.

(13) Chang, T. M. (2005) Therapeutic applications of polymeric artificial cells. *Nat Rev Drug Discov 4*, 221-35.

(14) Lushchak, V. I., and Bagnyukova, T. V. (2006) Effects of different environmental oxygen levels on free radical processes in fish. *Comp Biochem Physiol B Biochem Mol Biol. 144*, 283-9. Epub 2006 Jun 5.

(15) Eike, J. H., and Palmer, A. F. (2004) Effect of glutaraldehyde concentration on the physical properties of polymerized hemoglobin-based oxygen carriers. *Biotechnol Prog 20*, 1225-32.

(16) D'Agnillo, F., and Chang, T. M. (1998) Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. *Nat Biotechnol 16*, 667-71.

(17) Huang-Lee LL, C. D., Nimni ME. (1990) Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks. *J Biomed Mater Res. 24*, 1185-201.

(18) Gotshall RW, H. K., Foreman B, van Patot MC, Irwin DC. (2009) Glutaraldehyde-polymerized bovine hemoglobin and phosphodiesterase-5 inhibition. *Crit Care Med. 37*, 1988-93.

(19) Alayash, A. I., Summers, A. G., Wood, F., and Jia, Y. (2001) Effects of glutaraldehyde polymerization on oxygen transport and redox properties of bovine hemoglobin. *Arch Biochem Biophys 391*, 225-34.

(20) Winslow, R. M. (2004) MP4, a new nonvasoactive polyethylene glycol-hemoglobin conjugate. *Artif Organs 28*, 800-6.

(21) Manjula, B. N., Tsai, A. G., Intaglietta, M., Tsai, C. H., Ho, C., Smith, P. K., Perumalsamy, K., Kanika, N. D., Friedman, J. M., and Acharya, S. A. (2005) Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated through thiolation: influence on hemoglobin structure and function. *Protein J 24*, 133-46.

(22) Vandegriff, K. D., Malavalli, A., Wooldridge, J., Lohman, J., and Winslow, R. M. (2003) MP4, a new nonvasoactive PEG-Hb conjugate. *Transfusion 43*, 509-16.

(23) Hu, T., Li, D., Manjula, B. N., and Acharya, S. A. (2008) Autoxidation of the site-specifically PEGylated hemoglobins: role of the PEG chains and the sites of PEGylation in the autoxidation. *Biochemistry 47*, 10981-90.

(24) Chae, S. Y., Kim, S. W., and Bae, Y. H. (2002) Effect of cross-linked hemoglobin on functionality and viability of microencapsulated pancreatic islets. *Tissue Eng 8*, 379-94.

(25) Chae, S. Y., Kim, Y. Y., Kim, S. W., and Bae, Y. H. (2004) Prolonged glucose normalization of streptozotocin-induced diabetic mice by transplantation of rat islets coencapsulated with crosslinked hemoglobin. *Transplantation. 78*, 392-7.

(26) Barany, G., and Zalipsky, S. (1990) Facile synthesis of a-hydroxy-v-carboxymethylpolyethylene oxide. *Journal of Bioactive and Compatible Polymers 5*, 227-231.

(27) Zalipsky, S. (1995) Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates. *Bioconjug Chem 6*, 150-65.

(28) Iwasaki, K., Iwashita, Y., Ikeda, K., and Uematsu, T. (1986) Efficacy and safety of hemoglobin-polyethylene glycol conjugate (pyridoxalated polyethylene glycol hemoglobin) as an oxygen-carrying resuscitation fluid. *Artif Organs 10*, 470-4.

(29) Hu, T., and Su, Z. (2002) Preparation of well-defined bovine polyhemoglobin based on dimethyl adipimidate and glutaraldebyde cross-linkage. *Biochem Biophys Res Commun 293*, 958-61.

(30) Nonier, M. F., Vivas, N., Gaulejac de Vivas, N., and Fouquet, E. (2005) Development of direct size-exclusion chromatography separation for the determination of molar mass of native procyanidins in the phenolate form. *J Chromatogr A 1089*, 263-9.

(31) Patton, J. N., and Palmer, A. F. (2005) Photopolymerization of bovine hemoglobin entrapped nanoscale hydrogel particles within liposomal reactors for use as an artificial blood substitute. *Biomacromolecules 6*, 414-24.

(32) McCord, J. M., and Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem 244*, 6049-55.

(33) Flohe, L., and Otting, F. (1984) Superoxide dismutase assays. *Methods Enzymol 105*, 93-104.

(34) L'Abbe, M. R., and Fischer, P. W. (1990) Automated assay of superoxide dismutase in blood. *Methods Enzymol 186*, 232-7.

(35) Stern, K. G. (1937) On The Absorption Spectrum of Catalase. *J Biol Chem 121*, 561-572.

(36) Beers, R. F., Jr., and Sizer, I. W. (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem 195*, 133-40.

(37) Aebi, H. (1984) Catalase in vitro. *Methods Enzymol 105*, 121-6.

(38) Simoni J, S. G., Moeller JF. (2009) Intrinsic toxicity of hemoglobin: how to counteract it. *Artif Organs. 33*, 100-9.

(39) Talarico, T. L., Guise, K. J., and Stacey, C. J. (2000) Chemical characterization of pyridoxalated hemoglobin polyoxyethylene conjugate. *Biochim Biophys Acta 1476*, 53-65.

(40) Li, D., Manjula, B. N., Ho, N. T., Simplaceanu, V., Ho, C., and Acharya, A. S. (2007) Molecular aspects of the high oxygen affinity of non-hypertensive hexa pegylated hemoglobin, [(SP-PEG5K)(6)-Hb]. *Artif Cells Blood Substit Immobil Biotechnol 35*, 19-29.

(41) Linberg R, C. C., Shum KL, Shorr RG. (1998) Hemoglobin based oxygen carriers: how much methemoglobin is too much? *Artif Cells Blood Substit Immobil Biotechnol. 26*, 133-48.

(42) Vandegriff, K. D., Malavalli, A., Minn, C., Jiang, E., Lohman, J., Young, M. A., Samaja, M., and Winslow, R. M. (2006) Oxidation and haem loss kinetics of poly(ethylene glycol)-conjugated haemoglobin (MP4): dissociation between in vitro and in vivo oxidation rates. *Biochem J 399*, 463-71.

(43) Grey M, Y. S., Prachayasittikul V, Bülow L. (2009) A superoxide dismutase-human hemoglobin fusion protein showing enhanced antioxidative properties. *FEBS J. 276*, 6195-203.

(44) Veronese FM, C. P., Schiavon O, Sergi M. (2002) Polyethylene glycol-superoxide dismutase, a conjugate in search of exploitation. *Adv Drug Deliv Rev. 54*, 587-606.

(45) Veronese, F. M., Caliceti, P., Schiavon, O., and Sergi, M. (2002) Polyethylene glycol-superoxide dismutase, a conjugate in search of exploitation. *Adv Drug Deliv Rev 54*, 587-606.

(46) Yabe, Y., Nishikawa, M., Tamada, A., Takakura, Y., and Hashida, M. (1999) Targeted delivery and improved therapeutic potential of catalase by chemical modification: combination with superoxide dismutase derivatives. *J Pharmacol Exp Ther 289*, 1176-84.

(47) Simoni J, V. M., Simoni G, Moeller JF, Wesson DE. (2009) Control of oxidative reactions of hemoglobin in the design of blood substitutes: role of the ascorbate-glutathione antioxidant system. *Artif Organs. 33*, 115-26.

(48) CE., C. (2009) Radical producing and consuming reactions of hemoglobin: how can we limit toxicity? *Artif Organs. 33*, 110-4.

(49) Simoni J, S. G., Garcia EL, Prien SD, Tran RM, Feola M, Shires GT. (1995) Protective effect of selenium on hemoglobin mediated lipid peroxidation in vivo. *Artif Cells Blood Substit Immobil Biotechnol 23*, 469-86.

(50) Quebec, E. A., and Chang, T. M. (1995) Superoxide dismutase and catalase cross-linked to polyhemoglobin reduces methemoglobin formation in vitro. *Artif Cells Blood Substit Immobil Biotechnol 23*, 693-705.

(51) Kroemer, G., Marino, G., and Levine, B. (2010) Autophagy and the integrated stress response. *Mol Cell 40*, 280-93.

(52) Powanda, D. D., and Chang, T. M. (2002) Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. *Artif Cells Blood Substit Immobil Biotechnol 30*, 25-41.

(53) Halliwell, B., Clement, M. V., and Long, L. H. (2000) Hydrogen peroxide in the human body. *FEBS Lett 486*, 10-3.

(54) Funk, R. S., and Krise, J. P. (2007) Exposure of cells to hydrogen peroxide can increase the intracellular accumulation of drugs. *Mol Pharm 4*, 154-9.

3. EFFECTIVENESS OF HEMOGLOBIN CONJUGATES WITH ANTIOXIDANT ENZYMES IN PROTECTION OF PANCREATIC BETA CELL LINE FROM HYPOXIA

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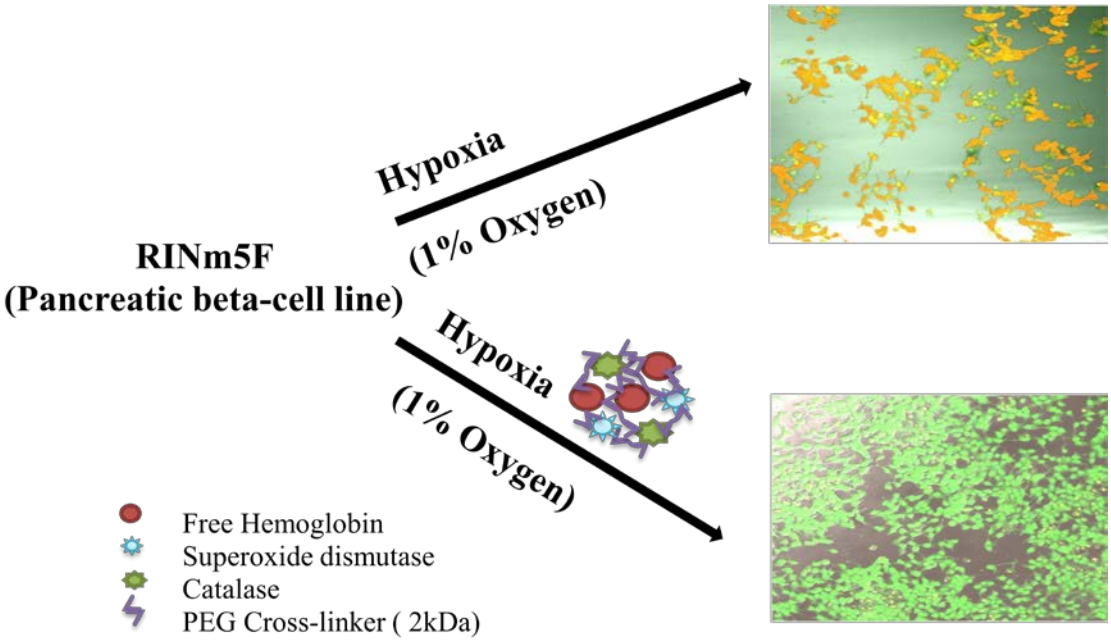
Nadithe, V., and Bae, Y. H. (2011) Hemoglobin conjugates with antioxidant enzymes (Hb-SOD-CAT) via poly(ethylene glycol) crosslinker for protection of pancreatic beta RINm5F cells in hypoxia. *Tissue Eng Part A.* July 11. http://www.liebertonline.com/doi/abs/10.1089/ten.tea.2010.0509

3.1 Abstract

This study investigated the effectiveness of using a low-p50 Hb-based O_2 carrier conjugated to antioxidants via a dicarboxymethylated poly(ethylene glycol) linker in protecting pancreatic beta cells from severe hypoxia at transplantation sites. The effectiveness was determined by calculating O_2 dissociation curves, Hill plots, Bohr coefficients, and the O_2 content of Hb conjugates. The protective effect due to incubation of Hb conjugates (Hb/PEG molar ratio 1:10) with pancreatic beta cells (RINm5F) against hypoxia (6%, 3%, and 1% O_2) was evaluated by MTT assay and confocal microscopy. The Hb conjugates with antioxidant enzymes were found to offer statistically significant protection (p < 0.01, increased viability ~80%) from hypoxia compared to control cells in a 1% O_2 environment. Confocal microscopy confirmed that the low-p50 Hb conjugates with antioxidants protected RINm5F cells from hypoxia (Scheme 3.1).



Scheme 3.1: Graphical Abstract

3.2 Introduction

Pancreatic beta cells are susceptible to damage by ROS under diabetic and hypoxic conditions, which can lead to islet dysfunction (*1-3*). Low O_2 pressure (generally between 40 and 5 mm Hg, corresponding to 0.5 to 6% of O_2 pressure), has been experimentally shown to exist in transplanted rat pancreatic islets, irrespective of the transplantation site (liver, kidney, or spleen) (*4*). The high sensitivity of islets to hypoxic stresses during islet isolation, culturing/maintenance, and posttransplantation is one of the major obstacles to successful islet transplantation (*5*). Lack of islet revascularization post-transplantation has also been shown to cause beta cell death *in vivo* (*6*). In one study, human islets and a beta cell-line (MIN6) under hypoxia (1% O_2) demonstrated loss of viability due to necrosis within 24h (*7*). Under these low-pO_2 conditions, hypoxic cell mitochondria also produce ROS, primarily due to altered cellular-redox potential caused by the slowing of respiratory chain reactions, which decreases the capacity of cytochrome C to trap O_2 (*8*). *In vivo*, tissues and cells with intact blood vessels sense changes in pO_2 through different mechanisms, and adapt to altered O_2 levels by altering ATP production, expressing hypoxia-induced factor (HIF) factor, increasing blood flow, and forming new blood vessels (*9*). Under hypoxic stress, intracellular pH may be decreased by the acidic environment (*10*) and the increased demand for O_2. Therefore, the design of an O_2 carrier that can release greater quantities of O_2 under conditions of low O_2 pressure would have great value in islet cell transplantation.

Most approaches used to design Hb-based artificial O_2 carriers synthesize Hb with properties similar to those of Hb inside RBCs using a typical sigmoidal cooperative O_2 binding. In perfluorocarbon (PFC)-based O_2 delivery systems, release of O_2 is of a linear rather than sigmoidal nature, in contrast to that of natural Hb. Low stability and PFC emulsion toxicity, as well as loose O_2 binding by van der Waals interactions compared to tight chemical bonding in natural Hb, are other significant shortcomings of PFC systems (*11*). These issues cause instantaneous release of O_2 by PFCs, and may not provide long-term benefits under hypoxia compared to low-p50 (high-affinity) O_2 systems (*12*).

To overcome the related problems of hypoxia and hypoxia-induced free-radical stress and/or environmental radicals, a suitable O_2 carrier providing a combined protective effect is highly desirable, specifically a multifunctional O_2 carrier of low p50 capable of releasing O_2 containing an inbuilt antioxidant defense mechanism that protects Hb from hypoxia-induced ROS and protects cells from hypoxia. Polymerization and cross-linking offer practical means of altering p50, depending on the cross-linking agent, and may be mediated by chemistry of the conjugation (*13*). A typical approach described in the literature using glutaraldehyde as a conjugating agent results in high p50, which is more relevant to normal conditions than the low p50 conditions encountered at transplantation sites (*14*). Moreover, glutaraldehyde cross-linking promotes vasoconstriction (*15*) and release of the polymeric linker by degradation, leading to cytotoxicity (*16*).

In a previous *in vitro* investigation of the protective effect of Hb–SOD–CAT at room temperature, the authors of this paper found that the antioxidant mechanism protected Hb from free radical stresses to a significant extent (*17*). The antioxidant enzyme SOD catalyzes the reduction of superoxide radicals to H_2O_2 and O_2 while CAT catalyzes the breakdown of H_2O_2 to H_2O and O_2. Research has shown that linking these enzymes to Hb by PEG only causes minimal loss of enzymatic activity postconjugation, with greater than 70% of SOD and 90% of CAT activity maintained (*17*). These enzymes may also provide a protective effect against the free radicals induced during hypoxia, and therefore help protect cells or tissues (*18*). In this study, O_2-binding and release capability was investigated to gain understanding of the means of tailoring low p50 Hb conjugates with antioxidant enzymes for specific tissue-engineering applications, such as protecting cells/tissues under hypoxia and/or hypoxia-induced free radical stress. To do so, this study examined the ability of these conjugates to protect a pancreatic beta cell line (RINm5F) under the hypoxic conditions of 6%, 3%, and 1% O_2 levels.

3.3 Experimental section

3.3.1 Materials

Bovine RBC suspension was purchased from Innovative Research (Novi, MI IC100-0410). Poly(ethylene glycol) (2 kDa); superoxide dismutase from bovine erythrocytes (SOD-S7571); bovine liver catalase (CAT-C40); cell culture media RPMI-1640 (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

(MTT); acridine orange (AO); propidium iodide (PI); potassium tertiary butoxide, ethyl bromoacetate, N-hydroxy-succinimide (NHS); and N,N′-dicyclohexylcarbodiimide (DCC) were purchased from Sigma Chemical Co. (St. Louis, USA). Penicillin-streptomycin, fetal bovine serum (FBS), and TrypsinEDTA-0.25% solution were purchased from Gibco Co. Dialysis membranes were purchased from Spectrum Labs (Rancho Dominguez, CA). Amicon™ Ultra-15 Centrifugal Filter Units, 50 and 100 kDa, were purchased from Millipore Corporation (Billerica, MA).

3.3.2 Conjugation of Hb with SOD and CAT

Hb was isolated from freshly collected bovine RBCs using the same procedure as that used in the previous experiment, and its purity verified employing SDS-PAGE and quantified employing the Drabkins method (*19*). PEG (2 kDa) was chemically modified as previously described(*19*); potassium tertiary butoxide and ethyl bromoacetate were used to modify the hydroxyl end groups on PEG to ethyl-protected carboxymethylated PEG. Basic hydrolysis of this intermediate PEG-compound resulted in a mixture of PEG-carboxylic acids. Further purification was performed by ion-exchange chromatography. PEG dicarboxylic acid used in conjugations was activated employing NHS and DCC. Bovine Hb was conjugated with and without SOD and CAT in various Hb:PEG conjugation ratios (1:5, 1:10, 1:15, 1:20, and 1:30), as previously described (*17*)**.** The ratio between SOD and CAT was fixed at 30000 to 300000 enzyme units in conjugation reactions with antioxidant enzymes. The conjugation reaction was

conducted by stirring for 3h at 4 °C and dialyzing the reaction product for 24h. Employing Amicon™ centrifugation filters, the desired concentration (1mM) of the conjugates was achieved. A 0.22-μm syringe was used to filter the product, which was stored at 4 °C until further use.

3.3.3 Measurement of O_2 equilibrium curves

The O_2 affinity of Hb-based conjugates was experimentally determined using the commercially available HEMOX Analyzer and HEMOX Analytical Software (TCS Scientific Corporation, New Hope, PA) (*20*). The O_2 equilibrium curve for both the O_2-association and dissociation phases was measured using dual-wavelength absorbance spectroscopy. A Clark electrode was used to directly measure the O_2 pressure in the sample cuvette. The OEC was obtained by plotting changes in optical absorbance on the X-axis and measuring maximum absorbance at 560 nm and 570 nm (the reference) at different pressures on the y-axis. For all experiments, 0.25 mM-equivalent Hb solution was placed in a cuvette containing the hemox solution, and the temperature of the mixture equilibrated to 37 °C by continuous heating and stirring while simultaneously being completely oxygenated using ultra-high pure O_2. A deoxygenation curve was obtained by replacing O_2 with ultra-high pure nitrogen gas in conditions in which Hb becomes deoxygenated ($HbO_2 \leftrightarrow Hb + O_2$). An oxygenation curve was obtained by replacing nitrogen with O_2 after the O_2 pressure had almost reached zero. All the samples are tested at three different pH values (6.5, 7.4, and 8.0) to determine their Bohr coefficients.

The OEC was obtained by stepwise curve-fitting analysis using the Adair equation, which assumes the sequential binding of O_2 to Hb at four available binding sites, each having a different equilibrium constant (*21*). The *y* values were calculated from the estimated Adair constants measured at each pressure and scaling the data points accordingly. The stepwise Adair Equation is as follows:

$$y = \frac{a_1 p + 2a_1 a_2 p^2 + 3a_1 a_2 a_3 p^3 + 4a_1 a_2 a_3 a_4 p^4}{4\left(1 + a_1 p + a_1 a_2 p^2 + a_1 a_2 a_3 p^3 + a_1 a_2 a_3 a_4 p^4\right)}$$

where *p* is O_2 pressure; *y* is the fractional O*2* saturation; and a1, a2, a3, and a4 are binding constants.

Hill plots were obtained by plotting $\mathrm{L}og\frac{y}{(1-y)}$ versus log (pO_2) values. The p50 value (the O_2 pressure at which the Hb molecule is 50% saturated) was calculated by extrapolating the x-axis value (oxygen partial pressure) to the y-axis value (Hb saturation). The Hill slope at p50 (n50) was obtained by calculating the slope at p50. Bohr coefficients were estimated using the Wyman equation (*22*), $\frac{\Delta \log \mathrm{P}_{50}}{\Delta pH}$, which estimates the average number of Bohr protons released (expressed as a negative value) per binding site upon full oxygenation.

3.3.4 Cell culture and hypoxia experiments

To estimate the effectiveness of Hb conjugates as O_2 carriers under hypoxic conditions (6%, 3%, and 1% O2), RINm5F cells (1×10^5 cells per well) were seeded using RPMI media containing 10% FBS and allowed to attach to plates by overnight incubation at 37 °C with 5% CO_2 and 95% air under normoxic conditions (21% O_2). After 24h, the medium was changed with fresh RPMI media containing Hb formulations with and without SOD and CAT. Based on our previous results showing low methemoglobin formation (a poor O_2 carrier), the optimized conjugating ratio was set at 1:10 Hb:PEG (*17*). The following groups were examined for the presence of Hb-conjugate activity: (1) cells with an RPMI medium only, (2) cells with an RPMI medium and 0.1 mM of unconjugated Hb, (3) cells with an RPMI medium and 0.1 mM of conjugated Hb-Hb, and (3) cells with an RPMI medium and 0.1 mM of Hb conjugated with antioxidants (Hb-SOD-CAT). Hypoxic conditions (6%, 3%, and 1% O_2) were achieved using a tri-gas incubator (Thermofisher Scientific Inc., Pittsburgh, PA, USA) with continuous injection of an appropriate quantity of ultra-high pure N_2 to reach target O_2 levels. At equilibrium, the incubator was saturated with the required percentage of O_2 (6% or 3% or 1%) and 5% CO_2, and then balanced with N_2. The cells were cultured for 48h in triplicate for each group by placing them in a hypoxic incubator.

3.3.5 O_2 content

The O_2 content at any given O_2 pressure in the medium was calculated using the following equation:

$$\text{Oxygen content } O_2 \ \frac{\text{mL}}{\text{L}} =$$

$$[\,(\text{Total Hb in } \frac{\text{grams}}{\text{L}}) \times (1.34) \times (\%\,\text{HbO}_2 \text{ expressed as fraction})\,] + [\,(pO_2 \text{ in mm Hg}) \times (0.03)\,]$$

where 1.34 mL of O_2 is bound by a gram of Hb (Hufner's number); the percentage of HbO_2 is equal to the quantity of O_2 combined with Hb; pO_2 is the pressure of dissolved O_2 in mm Hg; and 0.03 represents the conversion of 1 mm Hg of dissolved O_2 to mL/L (*23, 24*).

3.3.6 MTT assay of cell viability

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. After a defined treatment incubation period of 48h, MTT was added to the medium to obtain a final concentration of 0.5mg/ml, and then incubated at 37 °C under normoxic conditions (21% O_2) for 3h to allow for MTT reduction. The medium was aspirated and replaced with 1 mL of DMSO (dimethylsulfoxide) and incubated for another 15min to solubilize the formazan crystals. Absorbance at 570 nm was measured using a microplate reader (SpectraMax\M2; Molecular devices; Sunnyvale, CA). The absorbance values of all Hb-incubated samples were

normalized with the absorbance values of the cells growing in the medium only. The data shown in the MTT assay are relative cell viabilities expressed as means ± S.D. of three independent experiments.

3.3.7 Confocal microscopy

Apoptotic and dead cells were visualized using two fluorophores, acridine orange (AO) and propidium iodide (PI). The cells were incubated at room temperature for 15min and permeabilized with the dyes for detection of cell viability. Fluorescent images were obtained using the Fluoview confocal microscope (FV300, Olympus IX 81 microscope) by excitation of AO and PI at 500 and 536 nm, respectively, and emission at 530 and 620 nm, respectively. After sequential excitation, the green and red fluorescent images of the same cells were recorded and further image analysis performed using Image J software (http://rsbweb.nih.gov/ij/).

3.3.8 Statistical analysis

The statistical significance of the differences between the control group and the group of Hb-incubated formulations added to cells in hypoxic conditions were analyzed by ANOVA testing to compare group means. Multiple comparisons, including differences between polymer conjugation ratios, pH effect, and antioxidants, were formed by two-way ANOVA testing followed by Holm–Sidak testing. All $p < 0.05$ values were considered to be statistically significant. All the data are presented as means ± S.D.

3.4 Results

3.4.1 O_2 affinity of cross-linked Hb conjugates

The OEC of isolated bovine Hb at 37 ºC at different pH values (8.0, 7.4, and 6.5; Figure 3.1) shows the level of O_2 bounding. Bovine cell-free Hb has a p50 of 24.5 ± 0.6 mm Hg at normal physiological pH (7.4), similar to the value of 27.4 mm Hg reported in the literature (*25*). The corresponding values for pH 8.0 and 6.5 were 14.05 ± 0.35 and 38.66 ± 0.62 mm Hg, respectively, which is statistically significant ($p < 0.001$). These results demonstrate that the O_2 level in Hb solution is higher under basic conditions than acidic conditions at any given partial pressure. The difference also suggests that if cell-free Hb is placed in hypoxic tissues with pressure below 10 mm Hg (*26, 27*) (approximately 1 to 2% O_2), it will become saturated with up to 30%, 16%, and 10% O_2 at pH 8.0, 7.4, and 6.5, respectively, demonstrating that a greater quantity of O_2 is released in an acidic pH environment. This result also demonstrates that if pH decreases during hypoxia, the O_2 carrier may unload more O_2 for exchange with CO_2 to protect cells by decreasing acidity. Figure 3.2 indicates that at pH 7.4 and 37 $^{\circ}$C, there is a statistically significant ($p < 0.001$) decrease in p50 values by PEG-2kDa conjugation between Hb conjugates with and without SOD and CAT for all molar ratios compared to p50 of cell-free bovine Hb (the control).

A slight but statistically insignificant decrease in p50 was observed when varying the PEG ratio in the conjugates with and without SOD and CAT. It may be inferred from the results that increasing the concentration of PEG 2 kDa is

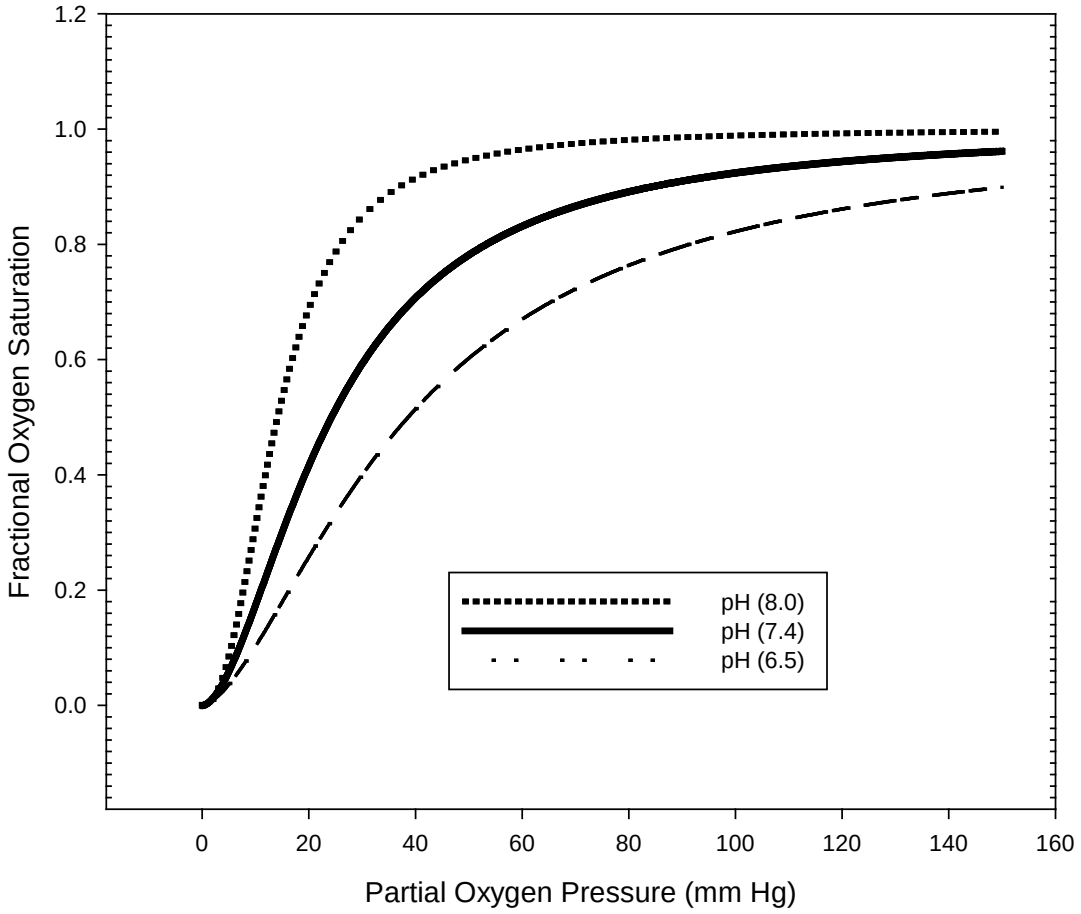


Figure 3.1. Fractional O_2 saturation curves of isolated free bovine Hb at different pH values (8.0, 7.4, and 6.5) and O_2 pressures.

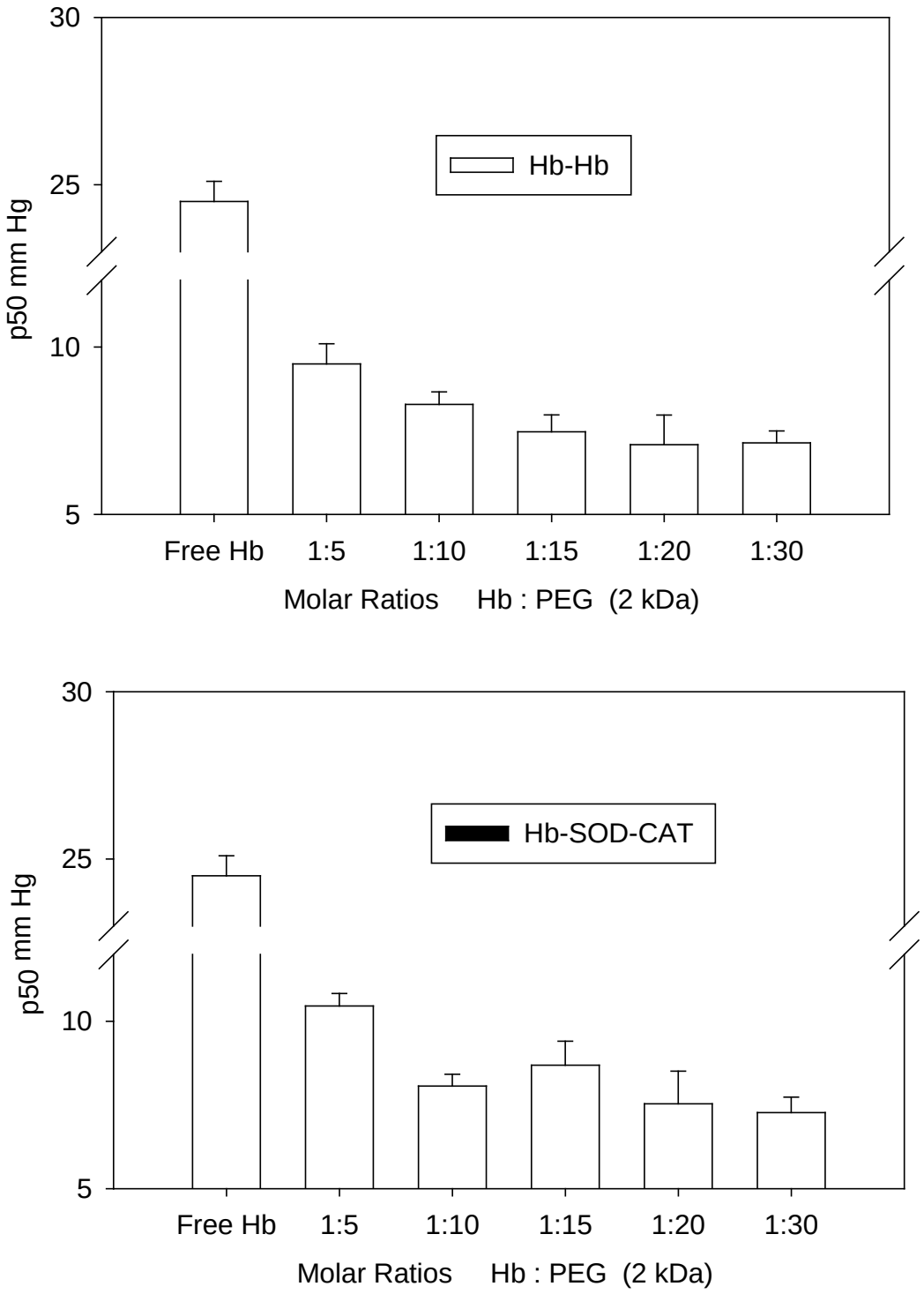


Figure 3.2. O_2 affinity of conjugated Hb with (Hb-SOD-CAT) and without (Hb-Hb) antioxidant enzymes at 50% of Hb saturation with O_2(p50) measured in pH 7.4.

not essential in further decreasing p50, and an optimum conjugation molar ratio was set. The results also show that the addition of antioxidant enzymes into the conjugation mixture did not significantly alter the p50 values. More significantly, the use of the conjugation method decreased the p50 values of the conjugates to ~7 to 10 mm Hg. The lowered p50 value (increased affinity) of these conjugates may increase the capacity of O_2 carriers to efficiently release higher amounts of O_2 under hypoxic conditions as compared to high-p50 cell-free Hb.

3.4.2 Hill coefficient and cooperativity

The Hill coefficient expresses the cooperative nature of Hb-O_2 binding. According to the theory of O_2 binding, the binding of one O_2 molecule in the heme pocket enhances the binding of subsequent O_2 molecules, thus yielding a typical sigmoidal O_2-binding curve with a positive Hill coefficient (*28*). The values of the Hill coefficients (Figure 3.3) (n_{50}-slope measured at p50) of isolated bovine Hb molecules at 37 $^{\circ}$C are 1.76 ± 0.17, 2.28 ± 0.10, and 1.61 ± 0.22 at pH 7.4, 8.0, and 6.5, respectively. The positive slope values of these pH conditions show that isolated Hb exhibits positive cooperative binding, as there is a direct positive correlation between the Hill coefficient and the extent of cooperative binding, with Hb molecules typically having a Hill coefficient of approximately 2 (*29*). The experimentally determined n_{50} values also show that Hb has a stronger cooperative effect under basic conditions than acidic conditions, indicating that using this conjugation method leads to a decrease in the cooperativity of Hb molecules.

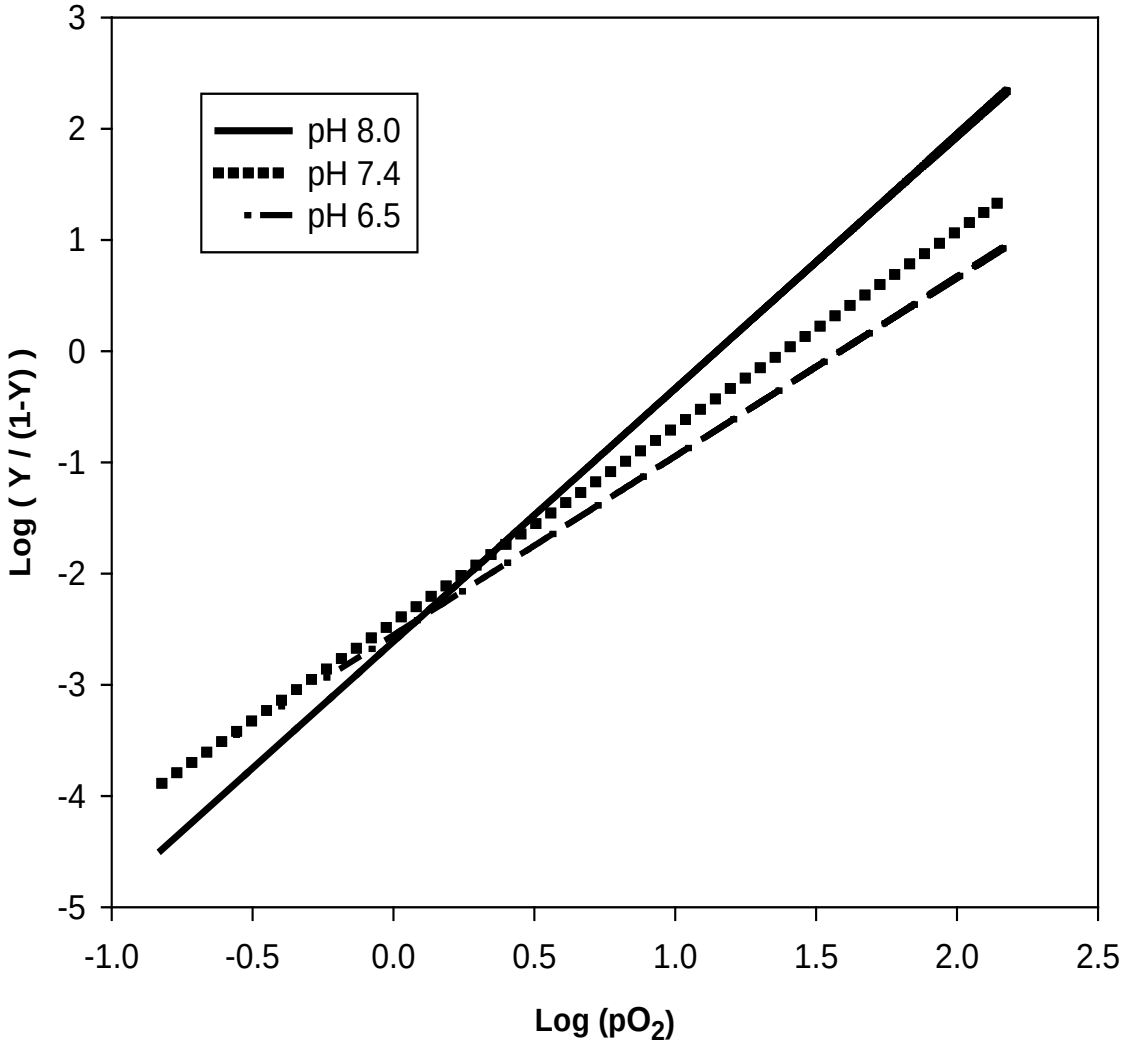


Figure 3.3. Hill plots of isolated free bovine Hb at different pH (8.0, 7.4, and 6.5) conditions.

The log p50 values (Figure 3.4) and their corresponding n_{50} values in Hb conjugate formulations with different PEG-2kDa molar ratios at three different pH values at 37 ºC show statistical significance ($p < 0.001$) when compared to the free Hb for all pH conditions. The n_{50} values range from 1 to 1.5 for all conjugates without SOD and CAT. Increasing the quantity of PEG 2 kDa for conjugation did not change the n_{50} value, indicating an insignificant change in the cooperative nature of O_2 binding among the conjugates. Figure 3.5 shows that the n_{50} values of Hb conjugated with SOD and CAT follow a trend similar to that of Hb conjugates without SOD and CAT. This finding implies that the addition of SOD and CAT does not have a significant effect on the cooperative nature of the O_2 binding of Hb within the conjugates. Therefore, it may be feasible to adjust the quantity of the antioxidant enzymes in the conjugates if a greater antioxidant effect is needed. The results show that the conjugation process did not significantly change the degree of positive cooperativity in the conjugated Hb, both with and without antioxidants. However, the positive cooperativity of Hb in Hb-Hb and Hb-SOD-CAT conjugates decreased significantly ($p < 0.001$) compared to that of free Hb. This decrease is also reflected in the altered p50 values in all the conjugates; at any given molar ratio, a very insignificant change in the cooperativity (n_{50}) in different pH conditions can be observed. However, increasing the molar ratio of PEG-2kDa led to only an insignificant decrease in the Hill coefficients, suggesting that the change in cooperativity with increased PEG has no significant effect compared to the cell-free Hb. The small change in

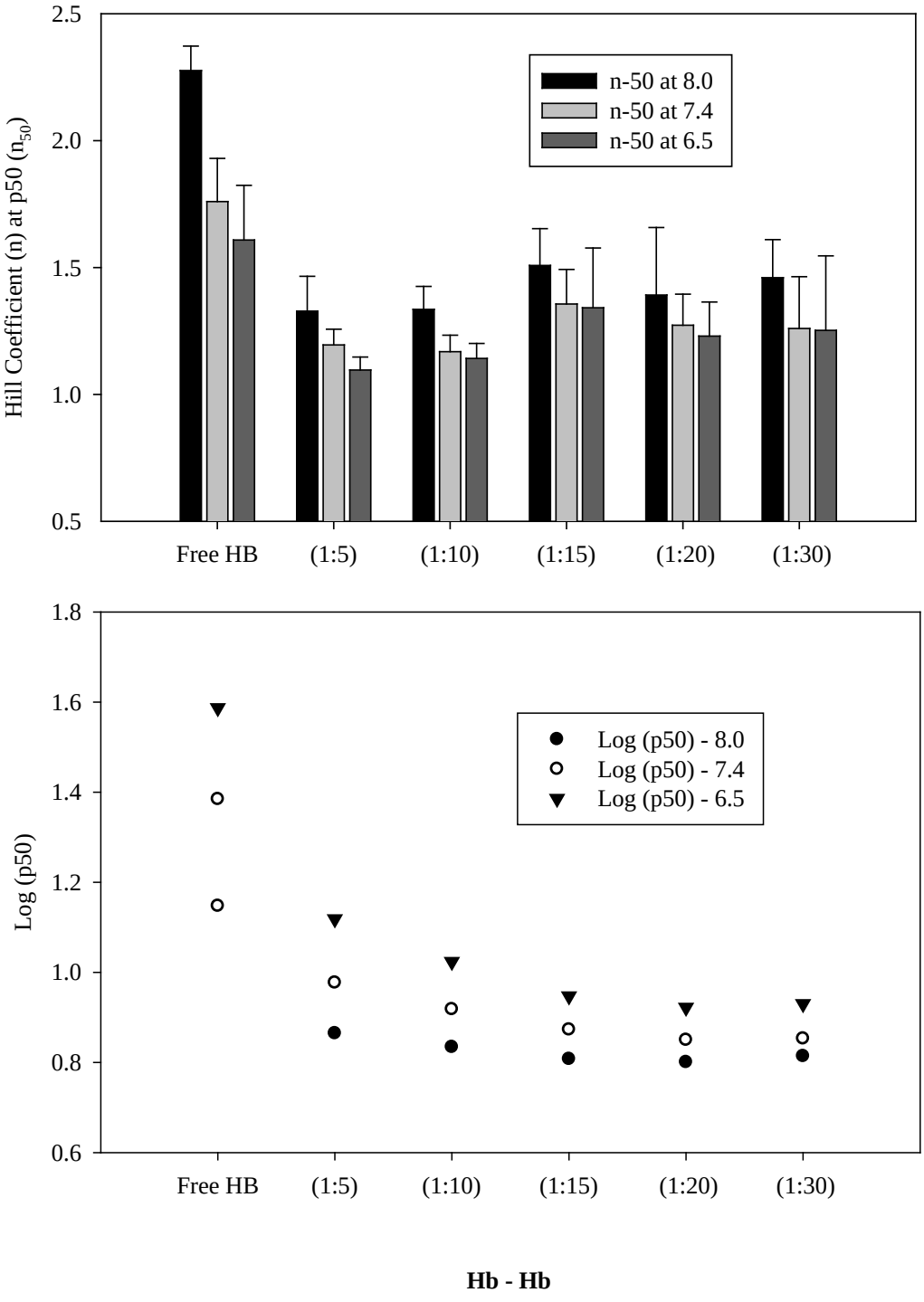


Figure 3.4. Hill coefficients (n_{50}) at corresponding log p50 of conjugated bovine Hb (Hb-Hb) for different Hb:PEG molar ratios (1:5, 1:10, 1:15, 1:20, 1:30) in different pH (8.0, 7.4, 6.5) conditions.

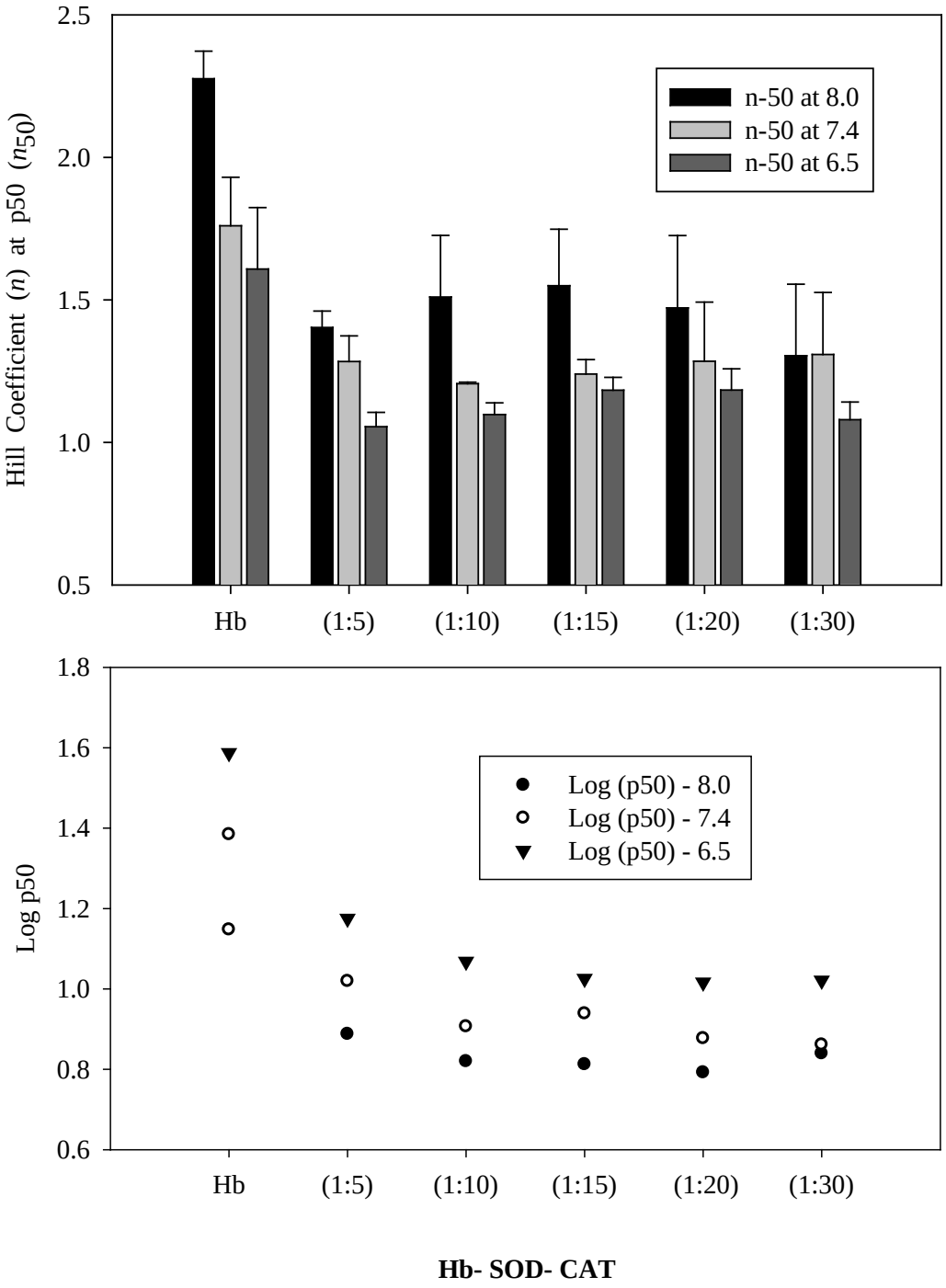


Figure 3.5. Hill coefficients (n_{50}) at corresponding log P_{50} of conjugated bovine Hb with antioxidant enzymes (Hb-SOD-CAT) for different Hb:PEG molar ratios (1:5, 1:10, 1:15, 1:20, 1:30) in different pH (8.0, 7.4, 6.5) conditions.

log p50 and the corresponding n_{50} value with pH change indicates that the conjugates may be less flexible in O_2 binding and release than is cell-free Hb.

3.4.3 Bohr effect

The Bohr effect is the impact of a change in pH on the O_2 affinity of a saturated oxyhemoglobin molecule. The effect originates from the conformational change in tetrameric Hb between the R (oxy) and T (deoxy) structure caused by the differences in binding and exchange between O_2 and H^+/CO_2 at the binding sites when there are changes in pH. The effect also leads to a change in the Hb molecular environment of Bohr groups (specific amino acid residues). The binding of O_2 to the heme groups decreases the pK_a of the Bohr groups and makes Hb more acidic, leading to the release of protons (*30*).

The Bohr effect is measured by the Bohr coefficient, which increases in tandem with increases in the quantity of O_2 that is off-loaded when a change in pH leads to a shift from an alkaline to an acidic condition (*31*). Isolated bovine Hb was found to have a negative Bohr coefficient of 0.30 (Figure 3.6) within the pH range (6.5 to 8.0) tested in this study, which is similar to the values reported in the literature (*30*). Conjugation with PEG decreased the absolute value of the Bohr coefficient by at least 50%, a statistically significant ($p < 0.001$) decrease compared to unconjugated free Hb that may have been due to alteration in Bohr groups in the Hb. Changing the quantity of PEG used in the cross-linking reaction mixture had no statistically significant effect on the Bohr coefficient.

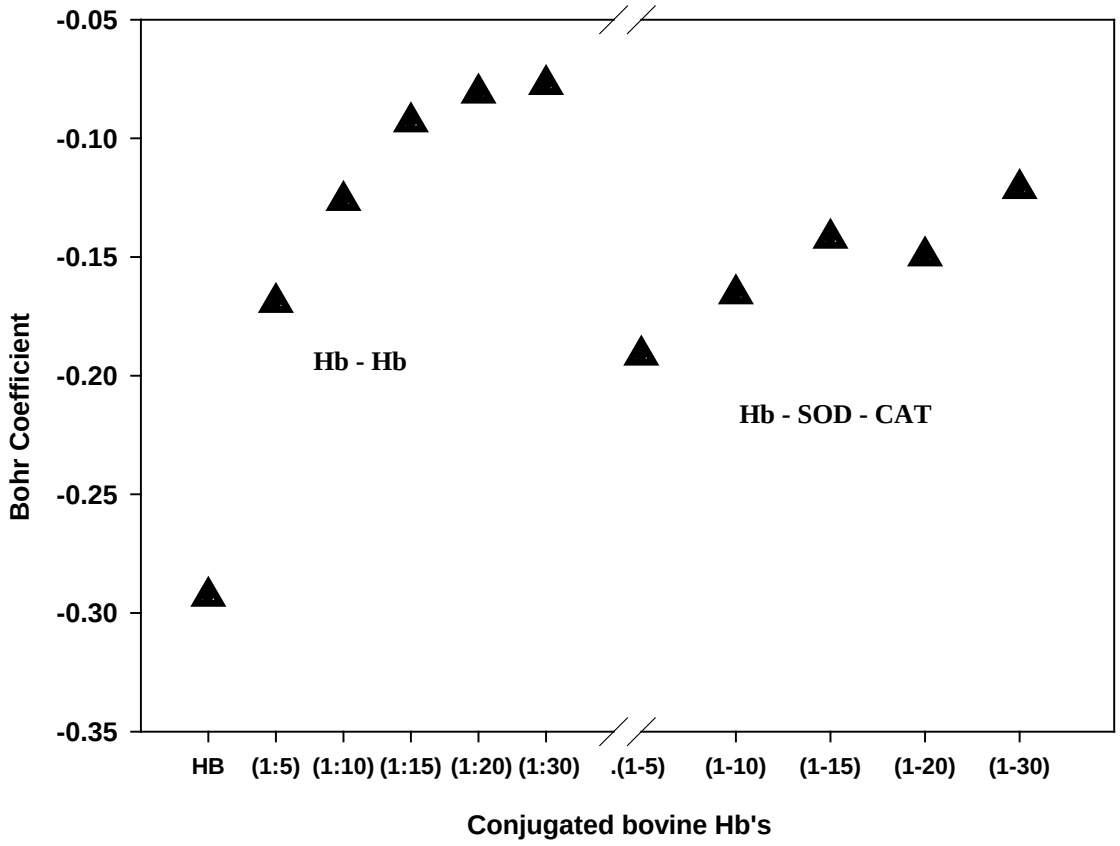


Figure 3.6. Bohr plot of conjugated bovine Hb with (Hb-SOD-CAT) and without (Hb-Hb) SOD and CAT at different Hb:PEG molar ratios (1:5, 1:10, 1:15, 1:20, 1:30).

The Bohr coefficients for cross-linked Hb indicate only a small change in O_2 off-loading with a change in pH from 6.5 to 8, demonstrating that cross-linked Hb are more susceptible to changes in O_2 pressure than a pH change from an alkaline to an acidic environment. The addition of SOD and CAT did not significantly change the Bohr coefficient compared to that of Hb-only conjugates.

3.4.4 O_2 content

The results displayed in Table 3.1 show that the total available O_2 is greater when p50 of Hb is lowered and calculated at different hypoxic conditions (6%, 3%, and 1%). At 40, 20, and 7 mm Hg of O_2 pressure, 1.2, 0.6, and 0.3 mL/L of soluble O_2, respectively, are available in the media for cellular respiration, values that are considerably low when compared to a normal O_2 value of 100 mm Hg (3 mL/L). Adding free Hb molecules (0.1mM, p50 ~24.5 mm Hg) to the media increases O_2 availability by 6.7 ± 0.2, 7.9 ± 0.2, and 1.8 ± 0.1 mL/L at 40, 20, and 7 mm Hg of O_2 pressure, respectively, while adding cross-linked Hb with and without SOD and CAT increases O_2 availability by 7.9 ± 0.2, 6.8 ± 0.3, and 5.2 ± 0.3 mL/L at 40, 20, and 7 mm Hg, respectively. These results show that by lowering the p50 of Hb, the percentage of O_2 will likely be increased by 18%, 79%, or 189% under a 6%, 3%, and 1% hypoxic condition, respectively, demonstrating the importance and usefulness of the low p50 conjugate.

3.4.5 Viability

MTT assays estimate cell viability by measuring the functional state of the mitochondria in cells. Viable cells with active mitochondria convert MTT to purple crystals (formazan) that can be dissolved in organic solvents and estimated spectrophotometrically. The data (Figure 3.7 and 3.8) demonstrate that cross-linked Hb-SOD-CAT (Hb/PEG 1:10 ratio) offers better cell protection in terms of percentage of relative RINm5F cell viability when incubated at 37 $^{\circ}$C

Table 3.1: Calculated O_2 content in Hb formulations (free Hb; conjugated Hb-Hb; conjugated with antioxidant enzymes Hb-SOD-CAT) and in media at different hypoxic conditions (6, 3, and 1% O2).

Percentage O_2	O_2 pressure	Hb formulation (0.1mM)	O_2 content (mL/L)	Percentage increase compared to free Hb	Soluble O_2 (mL/L)	Total O_2 content (mL/L)
6 %	~ 40 mm Hg	Hb	6.7 ± 0.2	*	1.2	7.9
		Hb-Hb	7.9 ± 0.1	18 %	1.2	9.1
		Hb-SOD-CAT	7.9 ± 0.2	18 %	1.2	9.1
3 %	~ 20 mm Hg	Hb	3.8 ± 0.1	*	0.6	4.4
		Hb-Hb	6.8 ± 0.3	79 %	0.6	7.4
		Hb-SOD-CAT	6.8 ± 0.2	79 %	0.6	7.4
1 %	~ 7 mm Hg	Hb	1.8 ± 0.1	*	0.21	2.0
		Hb-Hb	5.2 ± 0.2	189 %	0.21	5.4
		Hb-SOD-CAT	5.2 ± 0.3	189 %	0.21	5.4

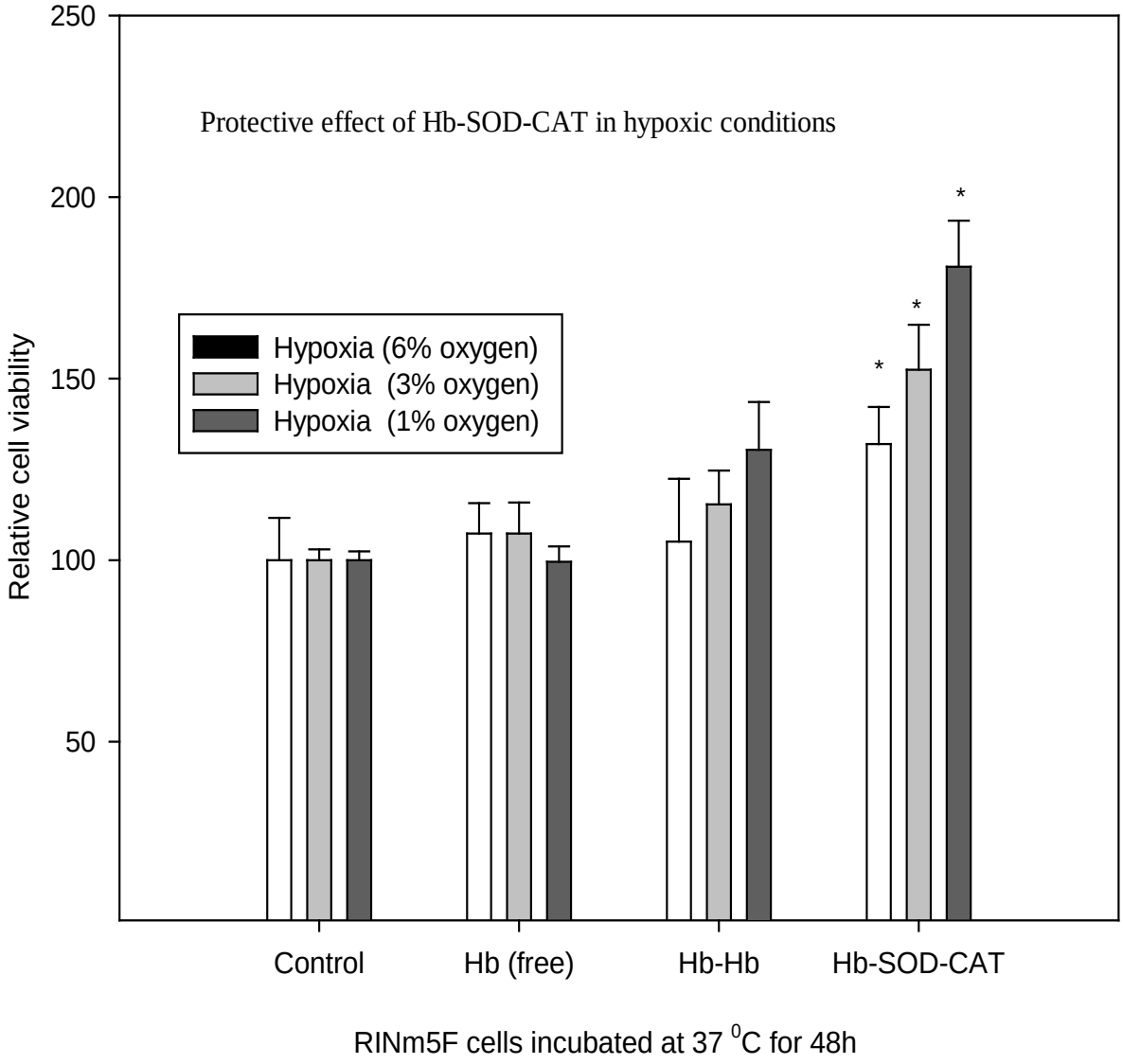


Figure 3.7. Percentage relative cell viability (MTT) of RINm5F cells incubated with RPMI media alone, free Hb, and conjugated bovine Hb with (Hb-SOD-CAT) and without (Hb-Hb) antioxidant enzymes (Hb:PEG 1:10) for 48h under hypoxic conditions (6%, 3%, and 1% O_2) (values = mean ± SD; n=3 * $p<0.01$). MTT values were normalized by the respective control cells in RPMI media under hypoxic conditions of 6%, 3%, or 1% O_2.

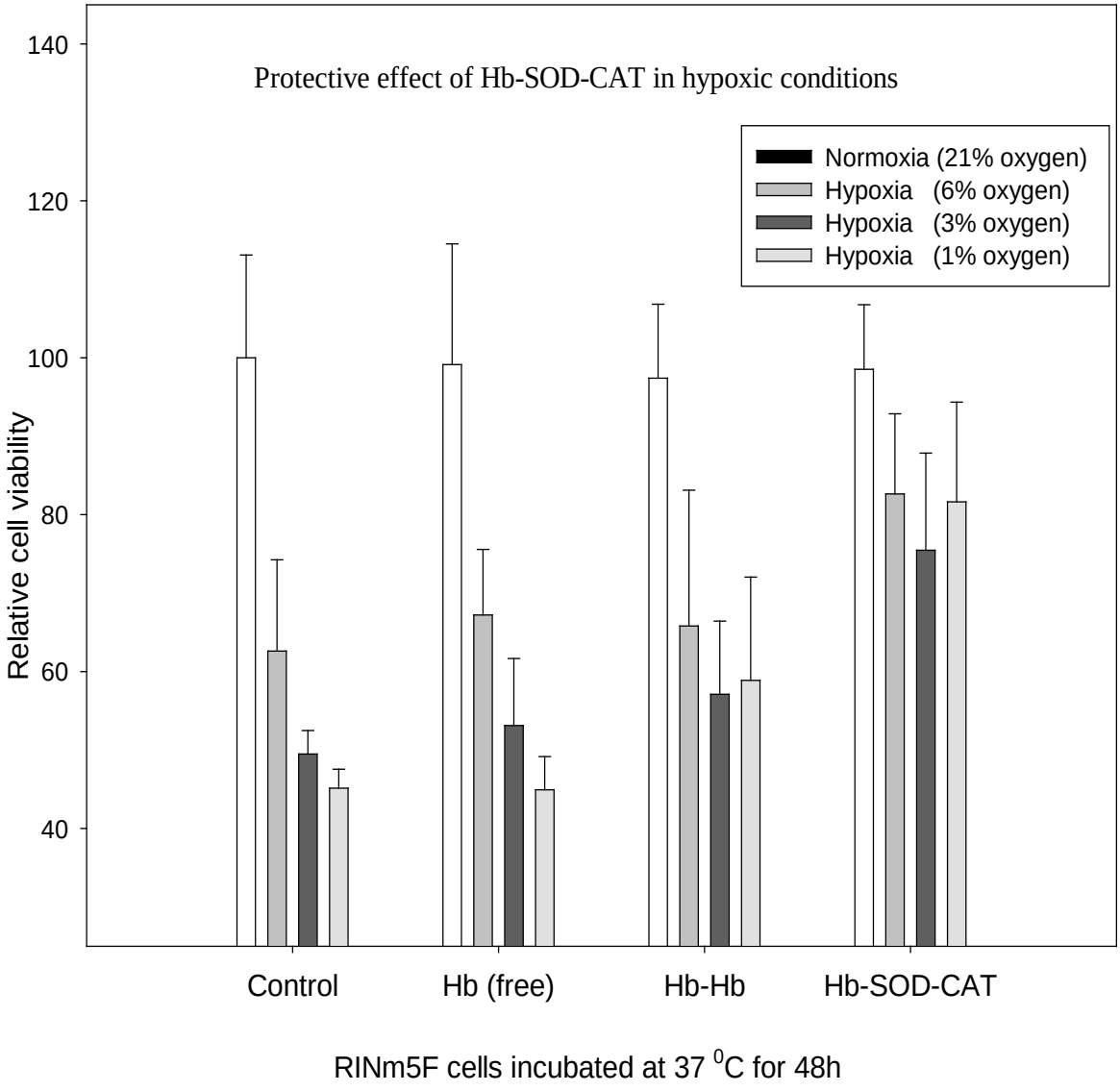


Figure 3.8. Percentage relative cell viability (MTT) of RINm5F cells incubated with RPMI media alone, free Hb, and conjugated bovine Hb with (Hb-SOD-CAT) and without (Hb-Hb) antioxidant enzymes (Hb:PEG 1:10) for 48h under normoxic conditions (21% O_2) and hypoxic conditions (6%, 3%, and 1% O_2) (values = mean ± SD; n=3). MTT values were normalized by control cells in RPMI media under a normoxic condition of 21% O_2.

under hypoxic conditions (6%, 3%, and 1% O_2) for 48h than do Hb conjugates without antioxidant enzymes.

The percentages of relative cell viability (Figure 3.7) under each hypoxic condition (6%, 3%, and 1% O_2) were calculated by normalizing MTT absorbance values with control cells growing in RPMI media alone. The use of cell-free Hb (p50 value of 24.5 mm Hg) demonstrated only a 5% to 10% higher cell viability compared to cells growing under hypoxic conditions, suggesting that it has a poor protective effect. The use of cross-linked Hb (Hb-Hb) (p50 value ~8 mm Hg) increased the viability by 5 ± 17%, 15 ± 9%, and 30 ± 13% under a 6%, 3%, and 1% hypoxic condition, respectively. When Hb was cross-linked with antioxidant enzymes with a similar p50 value of 8 mm Hg, the cell viability was further increased by 32 ± 10%, 52 ± 12%, and 81 ± 13% under a 6%, 3%, and 1% hypoxic condition, respectively. Statistical analysis demonstrated that the use of Hb with antioxidants provided a level of protection to RINm5F cells that was statistically greater than that provided by control cells with media alone ($p < 0.01$), free Hb ($p < 0.01$), and cross-linked Hb-Hb ($p < 0.05$). The use of cross-linked Hb with antioxidants appears more effective in 1% O_2 which is relevant to islet transplantation.

When the absorbance values were normalized by control cells under normoxic conditions (21% O_2) (Figure 3.8), the viability of cells growing in RPMI media alone under hypoxic conditions (6%, 3%, and 1% O_2) decreased by 63 ± 11%, 50 ± 3%, and 45 ± 2% under a 6%, 3%, and 1% hypoxic condition, respectively. The use of cell-free Hb (p50 value 24.5 mm Hg)

decreased viability by 66 ± 17%, 57 ± 9%, and 59 ± 13% under a 6%, 3%, and 1% hypoxic condition, respectively. The use of cross-linked Hb (p50 value ~8 mm Hg) decreased viability by 83 ± 10%, 75 ± 12%, and 82 ± 13% under a 6%, 3%, and 1% hypoxic condition, respectively.

3.4.6 Confocal microscopy

A combination of acridine orange (AO), a cell-permeable fluorescent dye that binds to DNA and emits green light when incorporated into normal healthy living cells, and propidium iodide (PI), a dye that emits red light when incorporated into the DNA of apoptotic or dead cells, to which it is permeable due to damage to the cell membrane, is used to qualitatively distinguish apoptotic from viable cells. Figures 3.9, 3.10, and 3.11 shows the confocal images of the RINm5F cells incubated with free Hb and Hb conjugates for 48h under hypoxic conditions. Control cells growing in 6%, 3%, and 1% O_2 demonstrated extensive cell membrane destabilization, leading to their uptake of PI. The decreased uptake of AO and the increased uptake of PI were greater in the control cells growing in 1% O_2, indicating the decreased viability of normal cells under harsh hypoxic conditions and the rapid death of cells incubated under hypoxic conditions without free and cross-linked Hb. The cells incubated in 6% O_2 with free and conjugated Hb demonstrated slightly higher cell viability (as observed in the MTT assay) compared to control cells, but no significant qualitative differences were found between the AO and PI uptake of the two groups of cells, and their confocal images appear to be similar.

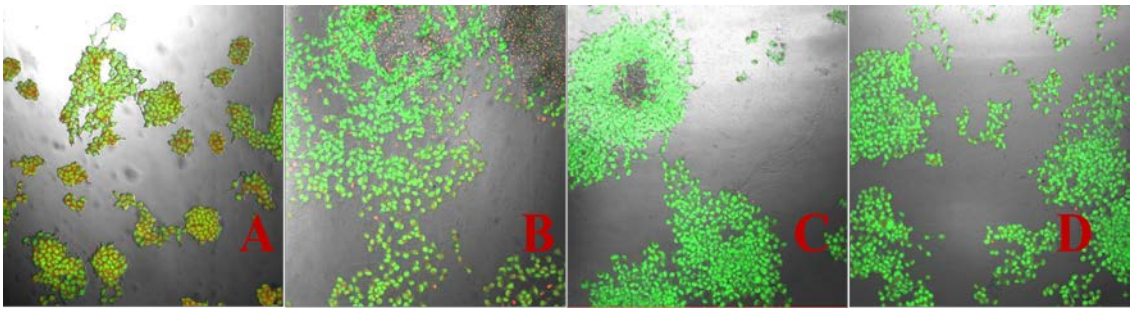


Figure 3.9. Confocal laser scanning microscopy images of RINm5F cells incubated with A) media only B) free Hb C) cross-linked Hb-Hb (Hb/PEG 1:10) and D) cross-linked Hb-SOD-CAT (Hb/PEG 1:10) for 48h under hypoxic conditions (6% O_2) (original magnification × 100).

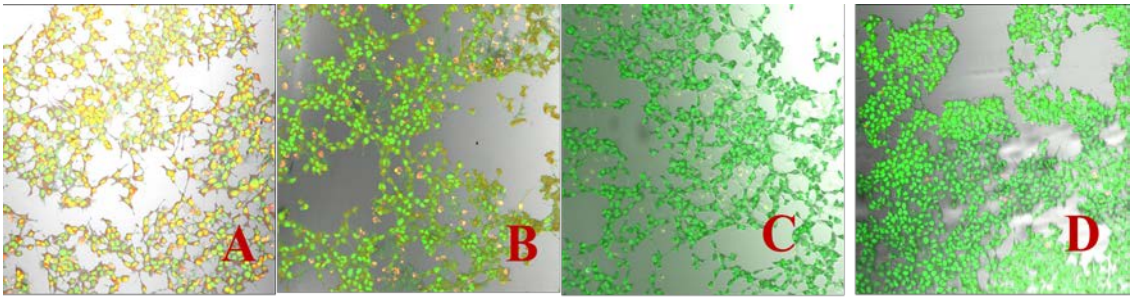


Figure 3.10. Confocal laser scanning microscopy images of RINm5F cells incubated with A) media only B) free Hb C) cross-linked Hb-Hb (Hb/PEG 1:10) and D) cross-linked Hb-SOD-CAT (Hb/PEG 1:10) for 48h under hypoxic conditions (3% O_2) (original magnification × 100).

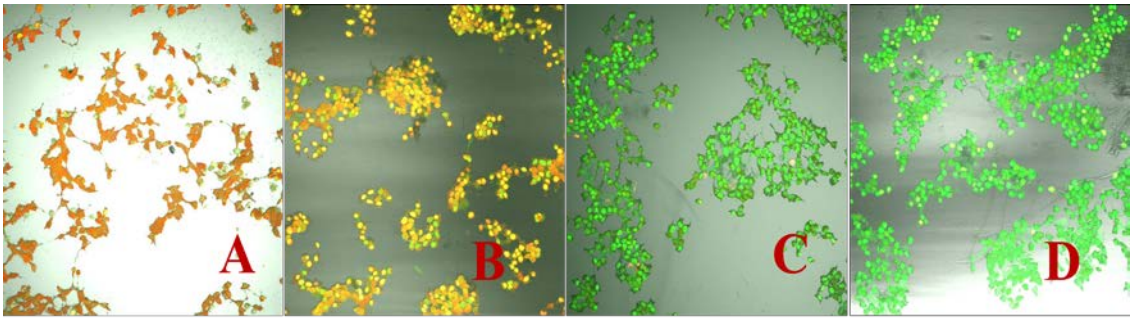


Figure 3.11. Confocal laser scanning microscopy images of RINm5F cells incubated with A) media only B) free Hb C) cross-linked Hb-Hb (Hb/PEG 1:10) and D) cross-linked Hb-SOD-CAT (Hb/PEG 1:10) for 48h under hypoxic conditions (1% O_2) (original magnification × 100).

However, the cells incubated in 3% O_2 with free Hb or conjugated Hb without SOD and CAT demonstrated significant PI uptake, indicating cell death.

The cells incubated with Hb conjugated with SOD and CAT demonstrated much higher viability. The cells growing in 1% O_2 and incubated with Hb-SOD-CAT were very permeable to AO, indicating a dramatic reduction in apoptosis. The results clearly demonstrate that Hb was effective in supplying O_2 from the conjugated Hb in severely hypoxic environments. More significantly, they suggest that low-p50 cross-linked Hb with antioxidants offers greater protection to RIN5mF cells from hypoxia-induced stress.

3.5 Discussion

The extent to which the efficiency of isolated Hb-based O_2 carriers can be improved depends primarily on the manner in which Hb is modified. When designing low-p50 O_2 carriers, the type of conjugating agent and the conjugation chemistry must be carefully considered, as they may significantly alter the sigmoidal shape of modified Hb, leading the shape of the resulting curve to differ from that of the typical OEC. This study found that employing glutaraldehyde, a commonly employed cross-linking agent that has been extensively reported in the literature, resulted in high p50 poly-Hb in 20:1 glutaraldehyde-Hb conjugation reactions. Although increasing the glutaraldehyde ratio to 40:1 decreased the p50 to 13 mm Hg, it also resulted in a significant conversion of Hb into methemoglobin (30%), potentially reducing

the role of the glutaraldehyde-Hb conjugation as an efficient O_2 carrier (*32*) under hypoxic conditions.

This study found that PEG-driven cross-linking significantly changed the shape of the OEC, and thus the quantity of O_2 released at different O_2 pressures, Hill coefficients, and Bohr coefficients. The O_2 carriers created by the PEG-conjugation chemistry used in this study decreased the p50 to ~8 mm Hg at a 10:1 PEG/Hb ratio and produced a minimal quantity of methemoglobin (< 5%)(*17*), suggesting that these O_2 carriers may function efficiently during islet-cell transplantation, especially under severe hypoxic conditions. A recent study found that the conjugation of PEG chains to Hb lysine residues by thiolation-mediated PEGylation induced structural changes by increasing the hydration shell, which further assisted in the stabilization of Hb into a desirable relaxed state (*33*). This stabilization into a relaxed state by increasing the PEG hydration shell or other modifications that aid in the transition of Hb from T to R conformation may be responsible for decreasing the p50 (*13*). However, using the current method of conjugation, it is not feasible to determine which amino group perturbation(s) is/are critical for decreasing the p50 of Hb molecules, and thus decreasing the p50 of the final conjugates (*34*).

Interestingly, this study found that increasing the concentration of PEG did not significantly decrease the p50, contrary to initial assumptions based on the literature (*32*). One explanation may be that amino groups (*34*) involved in the structural changes in the OEC at Hb:PEG molar ratios below 1:15 undergo complete modification. This study found only an insignificant change in the Bohr

coefficient of Hb, as well as that the addition of antioxidant enzymes did not significantly change the p50 of cross-linked Hb. The finding that there were no significant changes in O_2 release after pH changes in the tissue or cellular environment indicates that O_2 release by conjugated Hb is more susceptible to hypoxic conditions (O_2 levels less than 6%) than changes in cellular or media pH. The alterations in cooperativity and the Bohr coefficient that were observed, which are similar to those observed using most polymerization techniques (*35, 36*), may be attributed to the transformation of Glu-101 beta into acidic Bohr group which is in close proximity to Lys-99 alpha in oxyhemoglobin that may be involved in cross-linking (*37*).

For p50 of 24.5 mm Hg and n = 1.75, free Hb saturation was found to vary from 10% to 70% when the pO2 changed from 7 to 40 mm Hg (~1 to 6% O2). The saturation for cross-linked Hb-Hb or Hb-SOD-CAT was found to vary from 50 to 90% for p50 of 8 mm Hg and n = 1.2, indicating that cross-linking can effectively buffer Hb for cell transplantation under hypoxic conditions of 1 to 6% O_2. In the Hb in human RBCs, the T state (low O_2 affinity, high p50) is stabilized when 2,3 - BPG binds to deoxyhemoglobin by forming salt bridges with lysine and histidine residues (*38*). This causes the release of O_2 to surrounding tissues rather than its binding to Hb in the presence of 2,3-BPG and assists in oxygenation during tissue hypoxia.

It is important to note that bovine Hb, which was used in this study, does not behave in the same manner as 2,3-BPG allosterically, nor does myoglobin, as 2,3-BPG and has no effect on it (*39, 40*). It has been shown that the high O_2

affinity (low p50) and reduced cooperativity of PEG-modified Hb could lead to modification of four alpha-amino groups on Hb, as well as that the impact of lengthy PEG chains could lead to modification of the amino termini. Modification of val-1(α) only or of both Val-1(α) and Val-1(β) has also been shown to increase the O_2 affinity (low p50) of Hb. The stabilization of a high O_2 affinity has been explained as the impact of PEG on the Hb hydration shell. The epsilon-amino group of Lys-40(α), which forms a salt bridge with the alpha-carboxyl of His-146(β), may have played an important role in the cooperative O_2 binding by Hb observed in this study (*41*). However, the exact cause of the change in O_2 affinity due to the method used in this study cannot be determined at this stage.

Among the different conjugates that have been used in experiments under hypoxic conditions, this study used conjugates with a 1:10 (Hb:PEG) ratio in its MTT viability investigation. Molar ratios higher than 1:10 provide no additional benefit in terms of O_2 equilibrium properties, such as a low p50 value. The benefits of employing low-p50 systems in this study were similar to results observed by Sakai et al. when being compared to high-p50 systems (*42*). Although the O_2 content in low-p50 Hb conjugates with and without antioxidants was found to be similar under hypoxic conditions, the MTT results indicate greater cell viability in Hb conjugates with antioxidants. It was previously found that PEG-conjugated Hb (MP4) incubated at 37 ^{o}C for 18h produced 30 to 40% methemoglobin as the Hb underwent auto-oxidation (*43*). Based on previous research, this finding may be attributed to the protection of Hb by antioxidant enzymes in hypoxic environments from hypoxia-induced free-

radical stress and/or auto-oxidation, and thus greater cell viability, as indicated by the greater quantity of O_2 released in this study. As the conversion of Hb to methemoglobin is not instantaneous, the low-p50 Hb conjugate demonstrated greater cell viability than the free-Hb conjugate under severe hypoxic conditions (1% O_2). Despite the fact that the qualitative confocal images were similar for Hb conjugates with and without antioxidant enzymes, the differences in their cell viability may be the reason why RINm5F cells under normoxia multiply in a different manner from RINm5F cells under hypoxia (*44*).

The cells of cross-linked Hb in this study demonstrated 10 to 80% greater cell viability, depending on the severity of hypoxia, than did cells without any added Hb. The confocal microscopy results support the findings of the MTT assay. The significantly greater uptake of PI by the control RINm5F cells than of cells incubated with conjugated Hb clearly demonstrates the effectiveness of using conjugated Hb for O_2 transport under hypoxic conditions. Conjugated Hb may also be used for enhancing the survival of different cell or tissue types for temporary storage after isolation or during transplantation of beta or cardiac cells, as well as when treating ischemic conditions or related complications (*45-47*). For isolated beta-cell transplantation in low O_2 sites, such as the peritoneal cavity, exogenous low-p50 Hb conjugated with antioxidants may help prevent O_2 transport issues and reduce cell death from hypoxic and free-radical stresses. Due to the capacity of conjugated Hb to unload a greater quantity of O_2 into the cellular environment more efficiently under hypoxic conditions, and thus improve overall metabolic function, using conjugated Hb with low p50 in

such situations may be more beneficial than using high-p50 systems or other O_2 carriers (*48-52*).

3.6 Conclusion

The experiments performed in study demonstrated that the use of PEG-conjugated Hb with antioxidant enzymes can protect cells from hypoxia, a unique feature of low-p50 systems. The results reported here thus provide useful data for the optimization of O_2 carriers intended for therapeutic or prophylactic purposes under hypoxic and ischemic conditions. This study also described the design and use of a general approach for protecting isolated cells and tissues from combined hypoxia-induced oxidative and hypoxic stress.

3.7 Acknowledgments

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3.8 References

(1) Lee, S. C., Robson-Doucette, C. A., and Wheeler, M. B. (2009) Uncoupling protein 2 regulates reactive oxygen species formation in islets and influences susceptibility to diabetogenic action of streptozotocin. *J Endocrinol. 203*, 33-43. Epub 2009 Jul 27.

(2) Hou, N., Torii, S., Saito, N., Hosaka, M., and Takeuchi, T. (2008) Reactive oxygen species-mediated pancreatic beta-cell death is regulated by interactions between stress-activated protein kinases, p38 and c-Jun N-terminal kinase, and mitogen-activated protein kinase phosphatases. *Endocrinology. 149*, 1654-65. Epub 2008 Jan 10.

(3) Newsholme, P., Haber, E. P., Hirabara, S. M., Rebelato, E. L. O., Procopio, J., Morgan, D., Oliveira-Emilio, H. C., Carpinelli, A. R., and Curi, R. (2007) Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. *J Physiol. 583*, 9-24. Epub 2007 Jun 21.

(4) Carlsson, P. O., Palm, F., Andersson, A., and Liss, P. (2001) Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. *Diabetes. 50*, 489-95.

(5) Lau, J., Henriksnas, J., Svensson, J., and Carlsson, P.-O. (2009) Oxygenation of islets and its role in transplantation. *Curr Opin Organ Transplant. 14*, 688-93.

(6) Correa-Giannella, M. L., and Raposo do Amaral, A. S. (2009) Pancreatic islet transplantation. *Diabetol Metab Syndr. 1*, 9.

(7) Giuliani, M., Moritz, W., Bodmer, E., Dindo, D., Kugelmeier, P., Lehmann, R., Gassmann, M., Groscurth, P., and Weber, M. (2005) Central necrosis in isolated hypoxic human pancreatic islets: evidence for postisolation ischemia. *Cell Transplant 14*, 67-76.

(8) Paternotte, E., Gaucher, C., Labrude, P., Stoltz, J. F., and Menu, P. (2008) Review: Behaviour of endothelial cells faced with hypoxia. *Biomed Mater Eng 18*, 295-9.

(9) Chandel, N. S., and Budinger, G. R. (2007) The cellular basis for diverse responses to oxygen. *Free Radic Biol Med 42*, 165-74.

(10) Hilderbrand, S. A., Kelly, K. A., Niedre, M., and Weissleder, R. (2008) Near infrared fluorescence-based bacteriophage particles for ratiometric pH imaging. *Bioconjug Chem 19*, 1635-9.

(11) Riess, J. G. (2006) Perfluorocarbon-based oxygen delivery. *Artif Cells Blood Substit Immobil Biotechnol 34*, 567-80.

(12) Gardeazabal, T., Cabrera, M., Cabrales, P., Intaglietta, M., and Briceno, J. C. (2008) Oxygen transport during hemodilution with a perfluorocarbon-based oxygen carrier: effect of altitude and hyperoxia. *J Appl Physiol 105*, 588-94.

(13) Ronda, L., Bruno, S., Abbruzzetti, S., Viappiani, C., and Bettati, S. (2008) Ligand reactivity and allosteric regulation of hemoglobin-based oxygen carriers. *Biochim Biophys Acta. 1784*, 1365-77. Epub 2008 May 4.

(14) Powanda, D. D., and Chang, T. M. (2002) Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. *Artif Cells Blood Substit Immobil Biotechnol 30*, 25-41.

(15) Alayash, A. I., Summers, A. G., Wood, F., and Jia, Y. (2001) Effects of glutaraldehyde polymerization on oxygen transport and redox properties of bovine hemoglobin. *Arch Biochem Biophys 391*, 225-34.

(16) Huang-Lee LL, C. D., Nimni ME. (1990) Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks. *J Biomed Mater Res. 24*, 1185-201.

(17) Nadithe, V., and Bae, Y. H. (2010) Synthesis and characterization of hemoglobin conjugates with antioxidant enzymes via poly(ethylene glycol) cross-linker (Hb-SOD-CAT) for protection from free radical stress. *Int J Biol Macromol 47*, 603-13.

(18) Vazquez-Medina, J. P., Zenteno-Savin, T., and Elsner, R. (2006) Antioxidant enzymes in ringed seal tissues: potential protection against dive-associated ischemia/reperfusion. *Comp Biochem Physiol C Toxicol Pharmacol 142*, 198-204.

(19) Chae, S. Y., Kim, S. W., and Bae, Y. H. (2002) Effect of cross-linked hemoglobin on functionality and viability of microencapsulated pancreatic islets. *Tissue Eng 8*, 379-94.

(20) Guarnone, R., Centenara, E., and Barosi, G. (1995) Performance characteristics of Hemox-Analyzer for assessment of the hemoglobin dissociation curve. *Haematologica 80*, 426-30.

(21) Adair, G. S. (1925) The Hemoglobin System. VI. The Oxygen Dissociation Curve of Hemoglobin. *Journal of Biological Chemistry 63*, 529 - 545.

(22) Wyman, J., Jr. (1964) Linked Functions and Reciprocal Effects in Hemoglobin: a Second Look. *Adv Protein Chem 19*, 223-86.

(23) Irwin, D., Buehler, P. W., Alayash, A. I., Jia, Y., Bonventura, J., Foreman, B., White, M., Jacobs, R., Piteo, B., TissotvanPatot, M. C., Hamilton, K. L., and Gotshall, R. W. (2010) Mixed S-nitrosylated polymerized bovine hemoglobin species moderate hemodynamic effects in acutely hypoxic rats. *Am J Respir Cell Mol Biol 42*, 200-9.

(24) Muellenbach, R. M., Kredel, M., Zollhoefer, B., Wunder, C., Roewer, N., and Brederlau, J. (2006) Sustained inflation and incremental mean airway pressure trial during conventional and high-frequency oscillatory ventilation in a large porcine model of acute respiratory distress syndrome. *BMC Anesthesiol 6*, 8.

(25) Hu, T., and Su, Z. (2002) Preparation of well-defined bovine polyhemoglobin based on dimethyl adipimidate and glutaraldebyde cross-linkage. *Biochem Biophys Res Commun 293*, 958-61.

(26) Dimino, M. L., and Palmer, A. F. (2007) Hemoglobin-based O2 carrier O2 affinity and capillary inlet pO2 are important factors that influence O2 transport in a capillary. *Biotechnol Prog 23*, 921-31.

(27) Patton, J. N., and Palmer, A. F. (2006) Numerical simulation of oxygen delivery to muscle tissue in the presence of hemoglobin-based oxygen carriers. *Biotechnol Prog 22*, 1025-49.

(28) Yonetani, T., and Laberge, M. (2008) Protein dynamics explain the allosteric behaviors of hemoglobin. *Biochim Biophys Acta 1784*, 1146-58.

(29) Rameez, S., Alosta, H., and Palmer, A. F. (2008) Biocompatible and biodegradable polymersome encapsulated hemoglobin: a potential oxygen carrier. *Bioconjug Chem 19*, 1025-32.

(30) Barnikol, W. K. (1994) The proton Bohr factor of native and crosslinker treated hemoglobins--its possible significance for the efficacy of hemoglobin based artificial oxygen carriers. *Adv Exp Med Biol 361*, 363-70.

(31) Sasagawa, K., Imai, K., and Kobayashi, M. (2006) Influence of allosteric effectors and temperature on oxygen binding properties and the Bohr effect of bovine hemoglobin. *Zoolog Sci 23*, 565-72.

(32) Eike, J. H., and Palmer, A. F. (2004) Effect of glutaraldehyde concentration on the physical properties of polymerized hemoglobin-based oxygen carriers. *Biotechnol Prog 20*, 1225-32.

(33) Li, D., Manjula, B. N., Ho, N. T., Simplaceanu, V., Ho, C., and Acharya, A. S. (2007) Molecular aspects of the high oxygen affinity of non-hypertensive hexa pegylated hemoglobin, [(SP-PEG5K)(6)-Hb]. *Artif Cells Blood Substit Immobil Biotechnol 35*, 19-29.

(34) Pomponi, M., Gavuzzo, E., Bertonati, C., Derocher, A. E., Lydersen, C., Wiig, O., and Kovacs, K. M. (2004) Hemoglobin, pH and DPG/chloride shifting. *Biochimie 86*, 927-32.

(35) Hu, T., Li, D., and Su, Z. (2003) Preparation and characterization of dimeric bovine hemoglobin tetramers. *J Protein Chem 22*, 411-6.

(36) Rogers, M. S., Ryan, B. B., Cashon, R. E., and Alayash, A. I. (1995) Effects of polymerization on the oxygen carrying and redox properties of diaspirin cross-linked hemoglobin. *Biochim Biophys Acta 1248*, 135-42.

(37) Chatterjee, R., Welty, E. V., Walder, R. Y., Pruitt, S. L., Rogers, P. H., Arnone, A., and Walder, J. A. (1986) Isolation and characterization of a new hemoglobin derivative cross-linked between the alpha chains (lysine 99 alpha 1----lysine 99 alpha 2). *J Biol Chem. 261*, 9929-37.

(38) Berg JM, T. J., Stryer L. (2002) *Biochemistry*, 5 ed., W H Freeman, New York.

(39) Fronticelli, C. (1990) A possible new mechanism of oxygen affinity modulation in mammalian hemoglobins. *Biophys Chem 37*, 141-6.

(40) Gustin, P., Detry, B., Cao, M. L., Chenut, F., Robert, A., Ansay, M., Frans, A., and Clerbaux, T. (1994) Chloride and inorganic phosphate modulate binding of oxygen to bovine red blood cells. *J Appl Physiol 77*, 202-8.

(41) Hu, T., Prabhakaran, M., Acharya, S. A., and Manjula, B. N. (2005) Influence of the chemistry of conjugation of poly(ethylene glycol) to Hb on the oxygen-binding and solution properties of the PEG-Hb conjugate. *Biochem J 392*, 555-64.

(42) Sakai, H., Cabrales, P., Tsai, A. G., Tsuchida, E., and Intaglietta, M. (2005) Oxygen release from low and normal P50 Hb vesicles in transiently occluded arterioles of the hamster window model. *Am J Physiol Heart Circ Physiol 288*, H2897-903.

(43) Vandegriff, K. D., Malavalli, A., Minn, C., Jiang, E., Lohman, J., Young, M. A., Samaja, M., and Winslow, R. M. (2006) Oxidation and haem loss kinetics of poly(ethylene glycol)-conjugated haemoglobin (MP4): dissociation between in vitro and in vivo oxidation rates. *Biochem J 399*, 463-71.

(44) Guitart, A. V., Hammoud, M., Dello Sbarba, P., Ivanovic, Z., and Praloran, V. (2010) Slow-cycling/quiescence balance of hematopoietic stem cells is related to physiological gradient of oxygen. *Exp Hematol 38*, 847-51.

(45) Biolo, A., Greferath, R., Siwik, D. A., Qin, F., Valsky, E., Fylaktakidou, K. C., Pothukanuri, S., Duarte, C. D., Schwarz, R. P., Lehn, J. M., Nicolau, C., and Colucci, W. S. (2009) Enhanced exercise capacity in mice with severe heart failure treated with an allosteric effector of hemoglobin, myo-inositol trispyrophosphate. *Proc Natl Acad Sci U S A 106*, 1926-9.

(46) Irwin, D. C., Foreman, B., Morris, K., White, M., Sullivan, T., Jacobs, R., Monnet, E., Hackett, T., TissotvanPatot, M. C., Hamilton, K. L., and Gotshall, R. W. (2008) Polymerized bovine hemoglobin decreases oxygen delivery during normoxia and acute hypoxia in the rat. *Am J Physiol Heart Circ Physiol 295*, H1090-H1099.

(47) Kim, H. A., Lee, B. W., Kang, D., Kim, J. H., Ihm, S. H., and Lee, M. (2009) Delivery of hypoxia-inducible VEGF gene to rat islets using polyethylenimine. *J Drug Target 17*, 1-9.

(48) Mandic, M., Todgham, A. E., and Richards, J. G. (2008) Mechanisms and evolution of hypoxia tolerance in fish. *Proc Biol Sci*.

(49) Standl, T. (2005) A new oxygen transport agent. *Haematologica 90*, 437-8.

(50) Tsai, A. G., Vandegriff, K. D., Intaglietta, M., and Winslow, R. M. (2003) Targeted O2 delivery by low-P50 hemoglobin: a new basis for O2 therapeutics. *Am J Physiol Heart Circ Physiol 285*, H1411-9.

(51) Winslow, R. M. (2004) MP4, a new nonvasoactive polyethylene glycol-hemoglobin conjugate. *Artif Organs 28*, 800-6.

(52) Winslow, R. M. (2005) Targeted O2 delivery by low-p50 hemoglobin: a new basis for hemoglobin-based oxygen carriers. *Artif Cells Blood Substit Immobil Biotechnol 33*, 1-12.

4. CROSS-LINKED HEMOGLOBIN WITH ANTIOXIDANT ENZYMES PROTECTS PANCREATIC BETA ISLETS FROM FREE RADICAL STRESSES AND EXTENDS THE ISLET VIABILITY

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4.1 Abstract

Cross-linked hemoglobins using dicarboxymethylated poly(ethylene glycol) with the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were designed for the protection of pancreatic beta-cells from combined free-radical and hypoxic (6% and 1% oxygen) stress in conditions similar to islet transplantation.

In this study, the RINm5F (cell line) and isolated pancreatic beta-cells with oxidants (1mM H_2O_2 or 1mM xanthine and 10mU/mL xanthine oxidase) were challenged and incubated with hemoglobin conjugates (0.1mM Hb-Hb or Hb-SOD-CAT) in normoxia (21%) and hypoxia (6 and 1%) oxygen. Absorption spectroscopy and methemoglobin assay were used to evaluate hemoglobin protection at 37 $^{\circ}$C for 24h. Intracellular free radical activity and cell viability in RINm5F cells were measured by DCFDA and MTT assay. Radioimmunoassay was used to quantify insulin release in isolated islets. Confocal microscopy was used to qualitatively evaluate cell viability.

Results show that Hb-SOD-CAT helped maintain glucose-induced insulin secretion in beta-cells and RINm5F cell viability from combined oxidative and hypoxic stress. The study also suggests that antioxidant enzymes will play a significant role in the protection of hemoglobin in the conjugate and thus the extended cell protection in free radical and hypoxia-induced stress. These results also show the potential of Hb-SOD-CAT to prevent beta-cell dysfunction associated with free-radical and hypoxic stress in transplantation conditions.

4.2 Introduction

Islet transplantation can circumvent the need for daily injections of exogenous insulin in patients with Type 1 insulin-dependent diabetes mellitus (*1*). Even though this approach has shown some success, its widespread use is limited by islet donor shortage and long-term islet viability (*2*). Cell encapsulation and the use of nonhuman islets for transplantation can solve the problems of human islets scarcity and the need for immune suppression (*3*). Unfortunately, the long-term viability of isolated devascularized islets following transplantation remains an issue for achieving successful, long-term normoglycemia (*4*). Factors such as islet damage by reactive oxygen species sustained immediately after islet isolation from the pancreas or during *ex vivo* cell culture (*5*), hypoxia (*6*)**,** hypoxia-induced free-radical damage (*7*), and low partial oxygen pressures at the transplantation sites (*8*) are other important factors contributing to the loss of beta-cell viability and further hindering successful encapsulated islet transplantation. The presence of low levels of anti-oxidative enzymes and the minimal antioxidant capability of beta cells necessitate an antioxidant defense mechanism (*9*) to protect isolated islet beta cells.

A protective agent(s)/system that can be transplanted along with the islets at the transplantation site is necessary if the combined problem of hypoxia and free-radical stress on beta cells is to be overcome. Literature and previous results (*10, 11*) have shown that PEG-stabilized hemoglobin is a successful artificial oxygen carrier that can help in supplying oxygen to isolated cells. However, the long-term use of these cross-linked hemoglobins when co-

encapsulated with islets is limited by the continuous conversion of hemoglobin to methemoglobin by auto-oxidation (*12, 13*) and free radical damage (*14*). To overcome the problem of free radical damage to hemoglobin, modified dicarboxymethylated (PEG) was synthesized and used it to link two antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), to hemoglobin, creating hemoglobin (Hb) conjugate system. Previous *in vitro* studies using the conjugate system (Hb-SOD-CAT) had shown that the hemoglobin was protected effectively from free-radical damage caused by the superoxide anion and from hydrogen peroxide damage at room temperature (*15*) . PEG as the conjugating agent aided in designing a low p50 hemoglobin oxygen carrier that could unload higher amounts of oxygen when exposed to low partial oxygen pressures or under hypoxic conditions. This design also helped protect isolated cells in severe hypoxic stress when conjugated with SOD and CAT (*16*).

In this research, the robustness of the hemoglobin conjugated with a SOD and CAT system in a hypoxic (6% or 1% oxygen) and free-radical-rich environment were further tested to determine its suitability for long-term protection at transplantation sites. The effectiveness of SOD and CAT in protecting oxygen-loaded hemoglobin was tested in normoxia (21%) and hypoxia (6% or 1%) using cell incubation conditions that were relevant to an *in vivo* transplantation environment. After being challenged with superoxide anions, the hydroxyl ion and free radicals generated by hydrogen peroxide overall cell viability, intracellular free radical activity of a pancreatic beta cell line (RINm5F) was evaluated. Insulin-secreting capability by the primary isolated pancreatic

beta-islets was also determined. Cells were incubated at normal (21%) or hypoxic (6% or 1%) oxygen levels for 24h at 37 °C. Confocal microscopy was used to visually examine and corroborate the effectiveness of the Hb-SOD-CAT conjugate in preserving RINm5F cells and isolated islet viability when subjected to both hypoxic and free radical stresses.

4.3 Experimental section

4.3.1 Material

Freshly pooled bovine red blood cell suspension was purchased from Innovative Research (Novi, MI IC100-0410). PEG (2 kDa), potassium tertiary butoxide, ethyl bromoacetate, N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), superoxide dismutase from bovine erythrocytes (SOD-S7571), bovine liver catalase (CAT-C40), hydrogen peroxide (H_2O_2), xanthine, xanthine oxidase (XO), acridine orange (AO), propidium iodide (PI), (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)), RPMI 1640 culture medium powder, bovine serum albumin (BSA), and Dulbecco's phosphate buffered saline (DPBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin-streptomycin, fetal bovine serum (FBS), trypsin/EDTA-0.25% solution and 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) were purchased from Invitrogen, Inc. (Carlsbad, CA). Dialysis membranes were purchased from Spectrum Labs (Rancho Dominguez, CA). Amicon Ultra™-15 centrifugal filter units (50 and 100 kDa) were purchase from Millipore Corporation

(Billerica, MA). A ^{125}I-Insulin radioimmunoassay (RIA) kit was purchased from MP Biomedicals, Inc. (Irvine, CA).

4.3.2 Conjugation of Hb with SOD and CAT

As described previously (*11*), the hydroxyl end groups on PEG (2 kDa) were chemically modified to ethyl-protected carboxymethylated PEG using potassium tertiary butoxide and ethyl bromoacetate. Basic hydrolysis of ethyl-protected carboxymethylated PEG resulted in a mixture of PEG mono- and dicarboxylic acids. Purified PEG dicarboxylic acid was obtained by ion-exchange chromatography. NHS and DCC were used to further activate the purified PEG dicarboxylic acid. The activated PEG, PEG-NHS (from diacid) was characterized by ^{1}H NMR spectroscopy and thin-layer chromatography prior to use in the conjugation reaction. Fresh hemoglobin was isolated from bovine red blood cells using previously described methods (*11, 15*) . The hemoglobin was quantified using Drabkin's method, and the purity was confirmed by SDS-PAGE (*11*). For the conjugate formulation containing hemoglobin only (Hb-Hb), isolated purified hemoglobin was cross-linked using activated PEG at a fixed weight ratio of Hb:PEG (1:10). For the conjugate formulation containing antioxidant enzymes, the two antioxidant enzymes (SOD and CAT) at a fixed ratio of 150000 to 300000 enzyme units were mixed with hemoglobin using the same conjugation ratio of Hb:PEG (1:10), as described previously (*15*). The reaction was carried out at 4 °C for 3h with constant stirring, and the product was further dialyzed against PBS for 24h using dialysis membranes (MWCO 100 kDa). The desired concentration

of the conjugates (0.1mM) was achieved by concentrating the formulation using Amicon™ ultracentrifugation filters. The product was filter-sterilized using a 0.22 µm syringe filter and stored at 4 °C until further use.

4.3.3 Absorption spectrum of the conjugated hemoglobins after being challenged with free radicals (peroxide and superoxide) in normoxia and hypoxia at 37 °C

To study the protective effect of the antioxidant enzymes on hemoglobin in the combination formulation (Hb-SOD-CAT) in a hypoxic environment at 37 °C, the Hb-Hb and Hb-SOD-CAT (Hb:PEG 1:10) conjugates (each containing the equivalent of 0.1mM hemoglobin) were tested by exposing conjugated formulations to superoxide anion generated by 1mM xanthine and 10 mU/mL xanthine oxidase and hydroxyl free radicals generated by 1mM hydrogen peroxide in 24-well plates and incubated for 24h. The qualitative analysis of free radical and hypoxia-challenged conjugated hemoglobin systems was evaluated by measuring the visible absorption spectrum of conjugated hemoglobins using a UV-visible spectrophotometer (SpectraMax, Molecular Devices; Sunnyvale, CA). At the end of the 24h period, the conjugates were scanned through the visible absorption spectrum from 450 nm to 750 nm at a rate of 2 seconds.

4.3.4 Methemoglobin quantification

The methemoglobin content in the conjugated samples that had been challenged with free radical and hypoxic stress for 24h was determined using

methods previously described (*17*) and summarized as follows: Sufficient amounts of the challenged conjugates were transferred into a UV-visible cuvette by diluting the sample such that the absorbance reading (A1) at 630 nm was less than one unit. The sample was allowed to react with 50 µL of potassium cyanide solution (1 part 10% KCN and 1 part PBS pH 7.5). After the samples were reacted for 5-10min, a second absorbance measurement (A2) was taken at 630 nm. The methemoglobin content was calculated using the equation below:

$$\text{Methemoglobin} = \frac{\text{A1- A2}}{3.7} \times \text{dilution factor}$$

wherein the two absorbance values (A1 and A2) were subtracted from each other and divided by the extinction coefficient value (3.7 cm mM^{-1}). To determine the total hemoglobin content in the challenged conjugated samples, 50 µL of 20% $K_3Fe(CN)_6$ was added to the sample to convert any remaining oxy- and deoxy-hemoglobin species to methemoglobin. After another 50 µL of 10% KCN was added, a third absorbance peak (A3) was measured at 540 nm. The hemoglobin content was calculated by dividing the A3 absorbance value by the cyanomethemoglobin extinction coefficient of 11 cm mM^{-1} and multiplying by the dilution factor. The percentage of methemoglobin in the sample was calculated by taking the ratio of total methemoglobin and hemoglobin and multiplying it by 100.

$$\text{Hemoglobin} = \frac{A3}{11} \times \text{dilution factor}$$

4.3.5 Hypoxic and oxidative stress

Hypoxic stress was induced using a tri-gas incubator (Thermofisher Scientific Inc., Pittsburgh, PA) with continuous flushing of ultra-high pure N_2 to obtain the target hypoxic oxygen levels (6% or 1% of O_2). At equilibrium, the incubator contained either 6% or 1% of O_2, 5% CO_2 and rest balanced with N_2. Normoxic conditions (21% O_2) were maintained using a mixture of 95% air and 5% CO_2.

Oxidative stress was induced either by superoxide anion generated by 1mM xanthine and 10 mU/mL xanthine oxidase system (*18*) , or by hydroxyl free radicals by using 1mM hydrogen peroxide (*19*) . For all oxidant-challenge cell experiments, RIN5mF cells or islets were cultured in the presence of oxidants for 24h in the incubator at 37 $^{\circ}$C with n = 3.

4.3.6 Experimental groups

Experimental groups consisted of optimized hemoglobin conjugates made using the conjugation ratio of 1:10 Hb:PEG. This ratio was chosen from the previous optimized formulation that had low methemoglobin formation (*15*) and was an efficient oxygen carrier in hypoxic conditions (*16*). The following groups were selected for demonstrating the protective activity of Hb-conjugates: (1) cells with medium only; (2) cells with medium containing 1mM H_2O_2 or 1mM xanthine and 10mU/mL xanthine oxidase; (3) cells with medium containing 0.1mM

conjugated Hb-Hb and 1mM H_2O_2 or 1mM xanthine and 10mU/mL xanthine oxidase; (4) cells with medium containing 0.1mM hemoglobin conjugated with antioxidants (Hb-SOD-CAT) and 1mM H_2O_2 or 1mM xanthine and 10mU/mL xanthine oxidase.

4.3.7 Cell culture (pancreatic islets and RINm5F cell line)

Pancreatic islets were either isolated from male Sprague-Dawley Rat (200-250 grams) using enzymatic collagenase digestion and discontinuous Ficoll density gradient separation techniques (*10, 11*) or purchased from Joslin Diabetes Center, Boston, MA. Upon isolation or receipt, islets were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a cell culture incubator maintained at 37 °C containing 95% air / 5% CO_2 at normal humidity. Isolated islets were cultured for 24 to 48h before use in experiments. The normalized islet equivalent (islet size of 100 µm) was used to account for the natural variability in islet size.

RINm5F cells (rat insulinoma cell line) were cultured in RPMI1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a cell culture incubator maintained at 37 °C containing 95% air / 5% CO_2 at normal humidity. Cells were harvested using 0.25% trypsin/10 mM EDTA. RINm5F cells were seeded (1×10^5 cells per well) in tissue culture plates and allowed to attach for 24h prior to experiments.

4.3.8 Measuring intracellular reactive oxygen species (ROS) levels

RIN5mF cells that were challenged with oxidants in the presence of conjugates were washed with Dulbecco's Phosphate Buffered Saline (DPBS) so that intracellular reactive oxygen species (ROS) could be detected. The cells were then incubated with 20 μM of the cell-permeant ROS indicator 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA, Invitrogen) prepared in 1mL PBS for 60 min at 37 °C. The nonfluorescent diacetate form (H2DCF-DA) is a nonpolar molecule that diffuses into RIN5mF cells and then undergoes hydrolysis by cellular esterases to become dichlorofluorescein (DCFH), a hydrophilic nonfluorescent compound that becomes trapped inside the cell. DCFH is further oxidized to a fluorescent derivate dichlorofluorescein (DCF) by intracellular free radicals. DCF fluorescence was measured in a plate reader with an excitation/emission wavelength of 485/530 nm (*20*).

4.3.9 MTT assay for viability

After incubating RIN5mF cells with oxidants and formulations for 24h, cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay (*21*). MTT was added to the wells at a final concentration of 0.5mg/ml and incubated at 37 °C under normal oxygen conditions for 3h. The medium was removed, and formazan crystals were solubilized by adding 1mL of dimethylsulfoxide (DMSO) and incubating the plates for 10min at 37 °C. Absorbance was measured at 570 nm using a microplate reader absorbance (SpectraMax\M2, Molecular devices; Sunnyvale, CA). The

data shown for the MTT assay are percentage cell viabilities expressed as means ± S.D. accumulated from three independent experiments.

4.3.10 Insulin secretion assay

Islets (50) that had been incubated with oxidants and conjugated hemoglobin in either hypoxia or normoxia and culture media were removed after 24h, carefully rinsed with PBS, and placed in Kreb's Ringers HEPES (KRH) solution containing Ca^{+2} and 15.6g/dL glucose (450G) and 0.5%w/v BSA (KRH = 4.74mM KCl, 1.19mM KH_2PO_4, 1.19mM $MgCl_2.6H_2O$, 2.54mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES) (*22*). After 60min the medium was collected and insulin content was analyzed by RIA technique as per manufacturer instruction (^{125}I-Insulin radioimmunoassay from MP Biomedicals, Inc). Insulin secretion was normalized to control islets incubated in medium only, and the percentage of insulin secretion is expressed as means ± S.D. n = 3.

4.3.11 Confocal microscopy

Isolated pancreatic beta-islets were placed in four-well LabTek chambers (VWR, Bridgeport, NJ) and were incubated with Hb conjugates and challenged with oxidants and then visually analyzed for cell viability using confocal microscopy. Combination of acridine orange (AO - stains live cells) and propidium iodide (PI - stains apoptotic cells) (100 µl AO/PI solution; 0.67 µg/ml AO and 75 µg/ml PI in PBS) dyes were islet permeabilized in the dark for 10min at room temperature prior to imaging (*23*). By exciting at 500 nm and 536 nm and

detecting emission at 530 nm and 620 nm for AO and PI, respectively, confocal images were obtained using the Fluoview confocal microscope (FV300, Olympus IX 81 microscope). Image J software (http://rsbweb.nih.gov/ij/) was used to analyze the images.

4.3.12 Statistical analysis

The statistical significance between the different experimental groups (control, oxidant, oxidant and Hb-Hb, oxidant and Hb-SOD-CAT) in different partial oxygen pressures was analyzed by analysis of variance to compare group means followed by Holm–Sidak test. $P < 0.05$ was considered to be statistically significant. All data are presented as means ± standard deviation.

4.4. Results

4.4.1 Visible absorbance changes and methemoglobin

Overall results indicate that following a 24h challenge with hydrogen peroxide or superoxide anion at 37 $^{\circ}$C, there was substantial protection of hemoglobin observed in conjugates cross-linked with antioxidant enzymes (Hb-SOD-CAT) compared to Hb-only (Hb-Hb) conjugates (Figures 4.1 and 4.2). When Hb-Hb and Hb-SOD-CAT were compared for protective effect following the hydrogen peroxide or superoxide anion challenge, the decrease in characteristic hemoglobin absorbance read at 540 nm and 575 nm was higher for Hb-Hb than for Hb-SOD-CAT. The characteristic methemoglobin peak absorbance differences at 630 nm were higher for Hb-Hb and lower for Hb-SOD-CAT

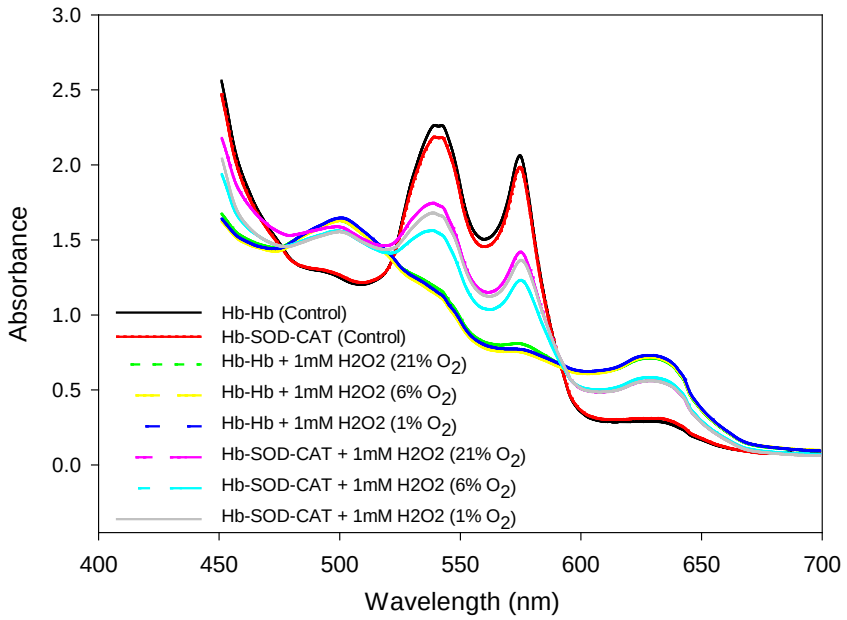

Figure 4.1: Absorbance spectra of hemoglobin conjugates incubated with 1mM H_2O_2 at 21%, 6% or 1% oxygen for 24h at 37 °C.

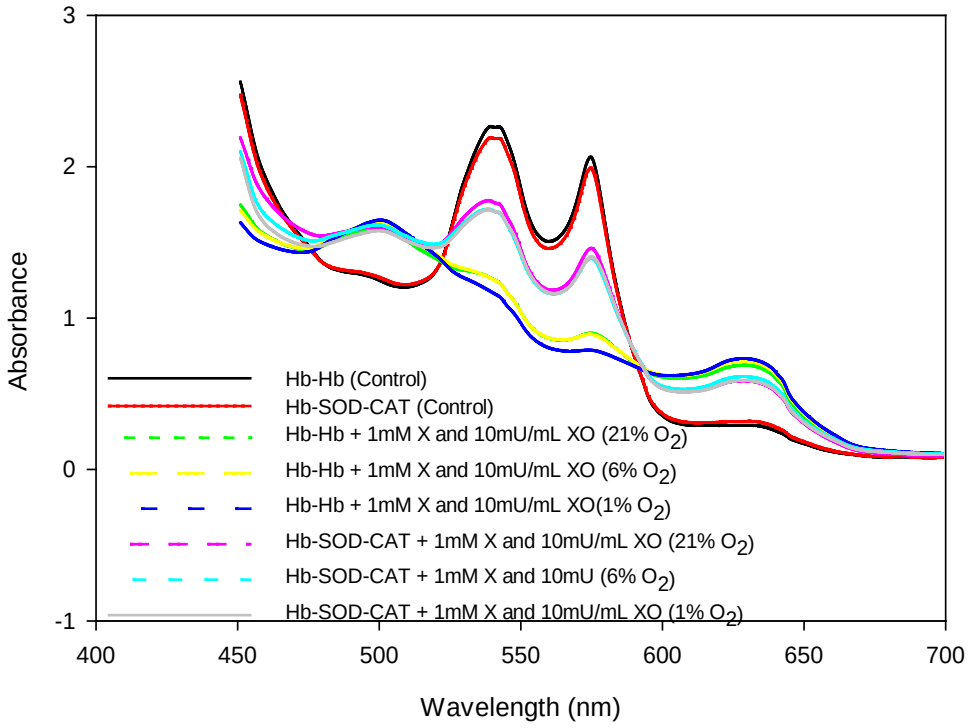

Figure 4.2: Absorbance spectra of hemoglobin conjugates incubated with 1mM xanthine and 10mU/mL xanthine oxidase at 21%, 6% or 1% oxygen for 24h at 37 °C.

between control and challenged conjugates at different partial (21%, 6%, and 1%) oxygen levels.

After a 24h incubation at 37 $^{\circ}$C with 1mM hydrogen peroxide, the percentage of methemoglobin found in Hb-Hb conjugates was 74 ± 2%, 71 ± 1%, and 72 ± 2% at 21%, 6%, and 1% oxygen levels, respectively, and 48 ± 3%, 52 ± 2%, and 50 ± 1% at 21%, 6%, and 1% oxygen levels for Hb-SOD-CAT (Figure 4.3). In this experiment, it was found that there were very few differences caused by different oxygen partial pressures, indicating that the oxygen content seems to have had a minimal effect in methemoglobin formation. In conjugates challenged with superoxide anion, the percentage methemoglobin formed in Hb-Hb was 62 ± 4%, 69 ± 3%, and 67 ± 1%; and for Hb-SOD-CAT conjugates the values were 44 ± 4%, 56 ± 2%, and 47 ± 1%, respectively, at 21%, 6%, and 1% oxygen levels (Figure 4.4). Statistical analysis showed significant differences in methemoglobin formation between Hb-Hb and Hb-SOD-CAT conjugates ($p < 0.01$) in 21%, 6%, and 1% oxygen. However, there was no statistical significance between the control conjugate samples and either peroxide or superoxide anion-challenged conjugates and incubated at the different oxygen partial pressures ($p > 0.5$). However, the results show an overall 20% lower methemoglobin formation when antioxidant enzymes were present in the cross-linked conjugate than the conjugates that did not contain antioxidant enzymes, at all different oxygen levels, and the results shows the usefulness of antioxidants.

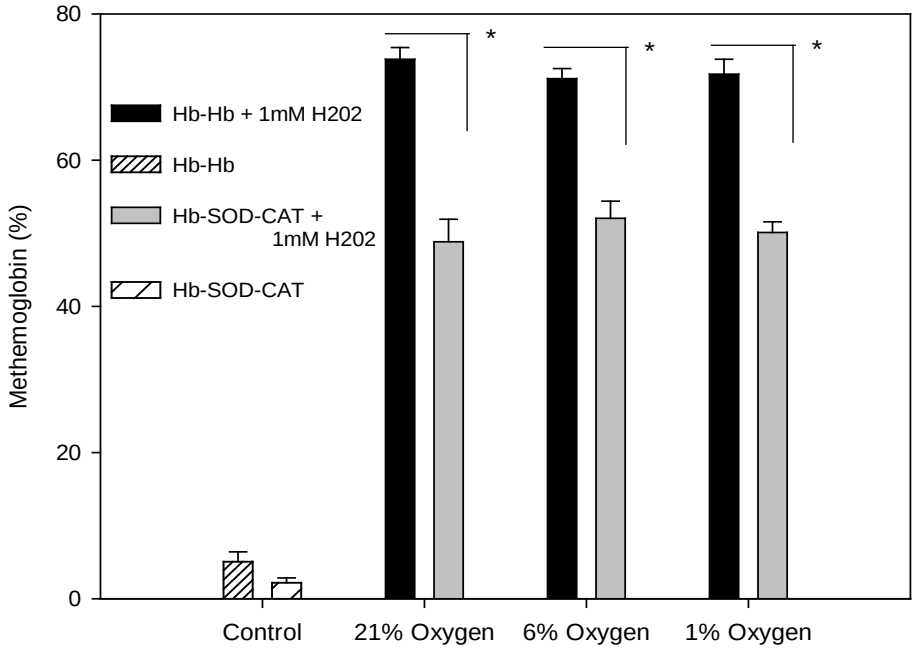

Figure 4.3: Methemoglobin content in hemoglobin conjugates incubated with 1mM H_2O_2 at 21%, 6% or 1% oxygen for 24h at 37 °C.

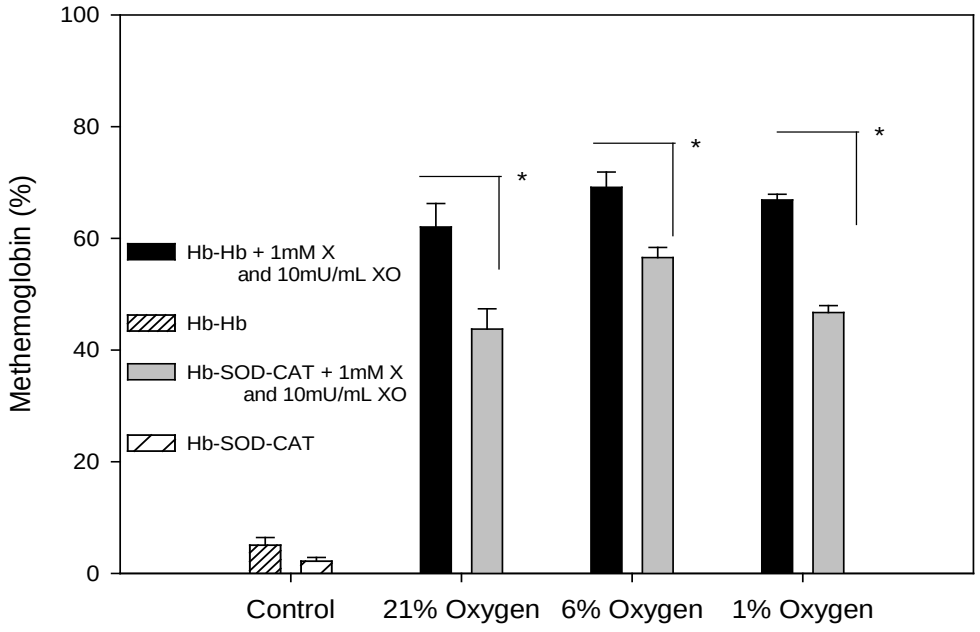

Figure 4.4: Methemoglobin content in hemoglobin conjugates incubated with 1mM xanthine and 10mU/mL xanthine oxidase at 21%, 6% or 1% oxygen for 24h at 37 °C.

4.4.2 Intracellular free radical activity

Intracellular free radical activity also can be measured to evaluate the capability of cells to adapt to external stressors or stimuli. When RINm5F cells were challenged with 1mM hydrogen peroxide and incubated with 0.1mM Hb-Hb, the relative fluorescence increased 16 ± 1%, 18 ± 1%, and 42 ± 6%; but when cells were incubated with 0.1mM Hb-SOD-CAT, the relative fluorescence increased only 3 ± 0.5%, 8 ± 1%, and 9 ± 1% (Figure 4.5). When the cells were incubated with 0.1mM Hb-Hb and challenged with superoxide anion, the relative fluorescence increased to14 ± 1%, 10 ± 1%, and 30 ± 3%; and when cells were incubated with 0.1mM Hb-SOD-CAT, the relative fluorescence increased only 3 ± 0.5%, 3 ± 0.5%, and 5 ± 1%; in oxygen tensions 21%, 6%, and 1%, respectively, compared to an untreated control incubated in media only (Figure 4.6). Statistical significant differences existed, when RINm5F cells were treated with peroxide, between cells incubated with media only (no conjugates; $p < 0.01$) and Hb-Hb ($p < 0.01$) or Hb-SOD-CAT ($p < 0.01$). For superoxide anion challenge, significant differences existed between cells incubated with media only (no conjugates; $p < 0.01$) and Hb-Hb ($p < 0.01$) or Hb-SOD-CAT at only 1% oxygen ($p < 0.01$). In addition, significant differences existed between Hb- Hb and Hb-SOD-CAT ($p < 0.01$), for both peroxide and superoxide anion, in terms of the increase in intracellular free radical activity at partial oxygen pressures of 21%, 6%, and 1% oxygen.

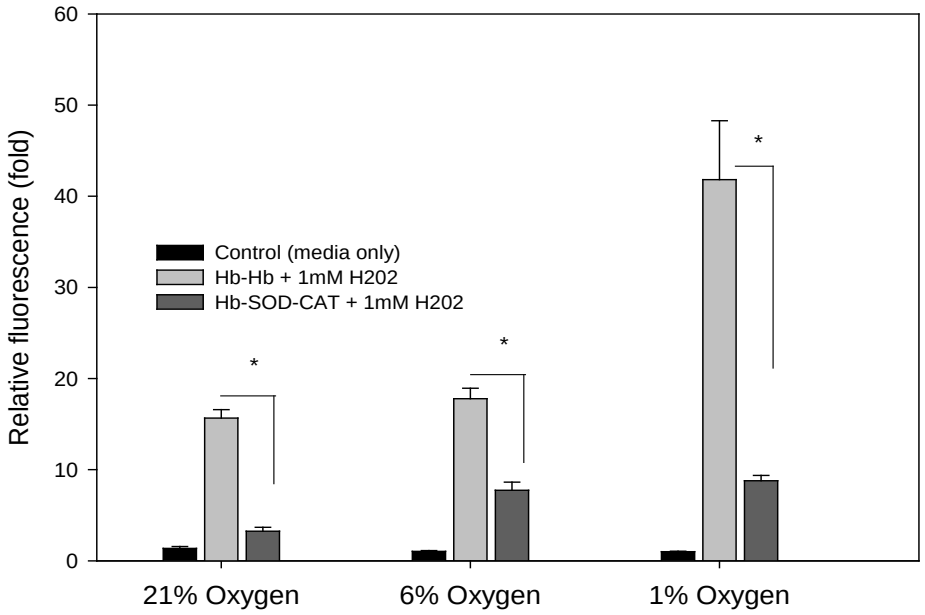

Figure 4.5: Intracellular free radical activity in RINm5F cells incubated with hemoglobin conjugates and challenged with 1mM H_2O_2 at 21%, 6% or 1% oxygen for 24h at 37 °C

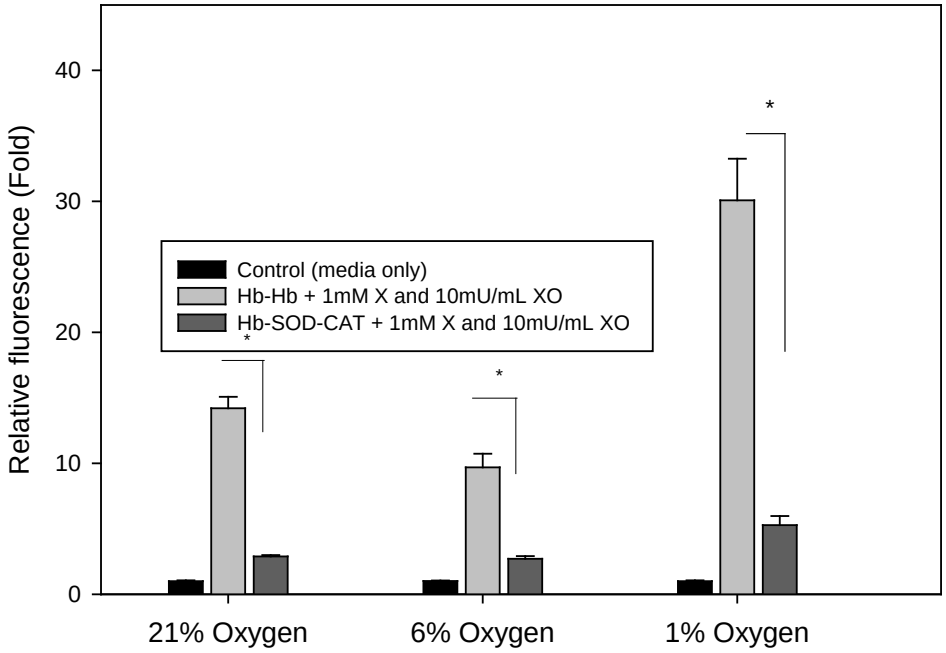

Figure 4.6: Intracellular free radical activity in RINm5F cells incubated with hemoglobin conjugates and challenged with 1mM xanthine and 10mU/mL xanthine oxidase at 21%, 6% or 1% oxygen for 24h at 37 °C.

4.4.3 Viability of RINm5F cells

The viability of RINm5F cells decreased from 100% (cells incubated in 21%) to 30 ± 10%, 18 ± 14%, and 20 ± 11% when challenged with 1mM hydrogen peroxide and incubated for 24 hours at 37 $^{\circ}$C at the different oxygen levels (21%, 6%, and 1%, respectively). Cells incubated with 1mM hydrogen peroxide and 0.1mM Hb-Hb without antioxidant enzymes at the different oxygen levels of 21%,6% and 1% had comparatively higher cell viabilities of 72 ± 15%, 73 ± 9%, and 59 ± 8%, respectively (Figure 4.7). However, cells incubated with 0.1mM Hb-SOD-CAT (with antioxidant enzymes) had cell viabilities of 96 ± 6%, 87 ± 10%, and 88 ±15% for oxygen pressures of 21%, 6%, and 1%, and are comparable substantially to control cells in media incubated in 21% oxygen and higher than Hb-conjugates without antioxidant enzymes. Statistical analysis showed that there are no significant differences ($p > 0.1$) in cell protection between control cells in 21% oxygen and cells challenged by 1mM hydrogen peroxide and incubated with antioxidant enzymes (Hb-SOD-CAT) at all tested partial pressures (21%, 6%, and 1%) of oxygen. Statistical significance is observed between control cells in 21% oxygen to cells treated with 1mM hydrogen peroxide ($p < 0.001$), and 1mM hydrogen peroxide treated and 0.1mM incubated Hb-Hb without antioxidant enzymes ($p < 0.05$) at partial pressures (21%, 6%, and 1%) of oxygen, indicating the inability of Hb-Hb to protect cells in combined free radical and hypoxic stress.

When RINm5F cells were incubated with superoxide anion generated by 1mM xanthine and 10mU/mL xanthine oxidase, cell viability decreased to 33 ±

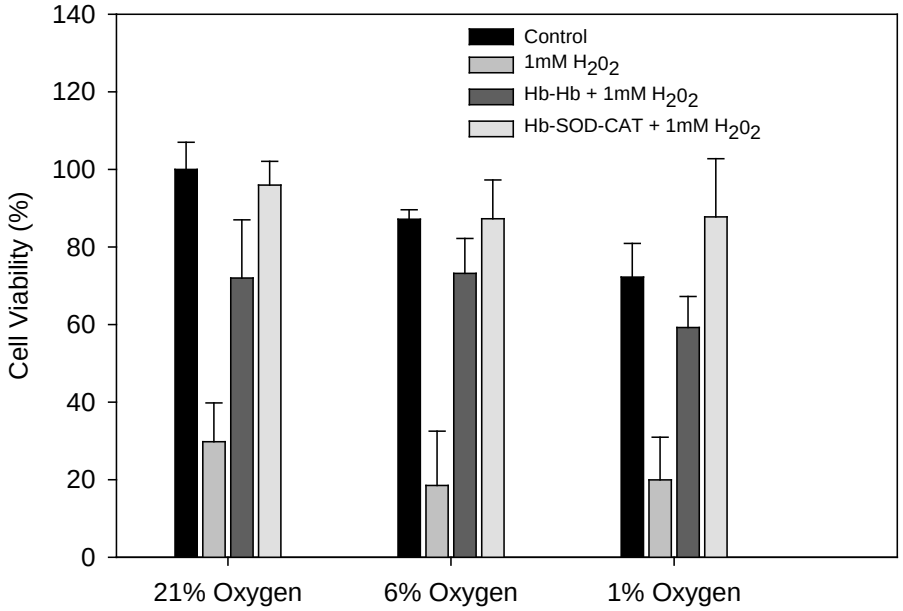

Figure 4.7: Relative cell viability of RINm5F cells incubated with hemoglobin conjugates and 1mM H_2O_2 at 21%, 6% or 1%) oxygen for 24h at 37 °C.

11%, 35 ± 17%, and 31 ± 9% for 21%, 6%, and 1% oxygen, respectively (Figure 4.8). Cells incubated with superoxide anion and 0.1mM Hb-Hb had cell viabilities of 73 ± 8%, 72 ± 18%, and 56 ± 21% for 21%, 6%, and 1% oxygen, respectively. When cells were challenged with superoxide anion and incubated with 0.1mM Hb-SOD-CAT (containing antioxidant enzymes), RINm5F cells maintained viabilities of 93 ± 12%, 86 ± 11%, and 87 ± 7% at oxygen content of 21% and 6% or 1% oxygen. Statistical analysis showed that there are no significant differences ($p > 0.1$) between control cells in 21% oxygen and cells challenged by superoxide anion and incubated with Hb-SOD-CAT at partial pressures (21%, 6%, and 1%) of oxygen, suggesting that antioxidant enzymes were successful in removing superoxide anion. Statistical significance is

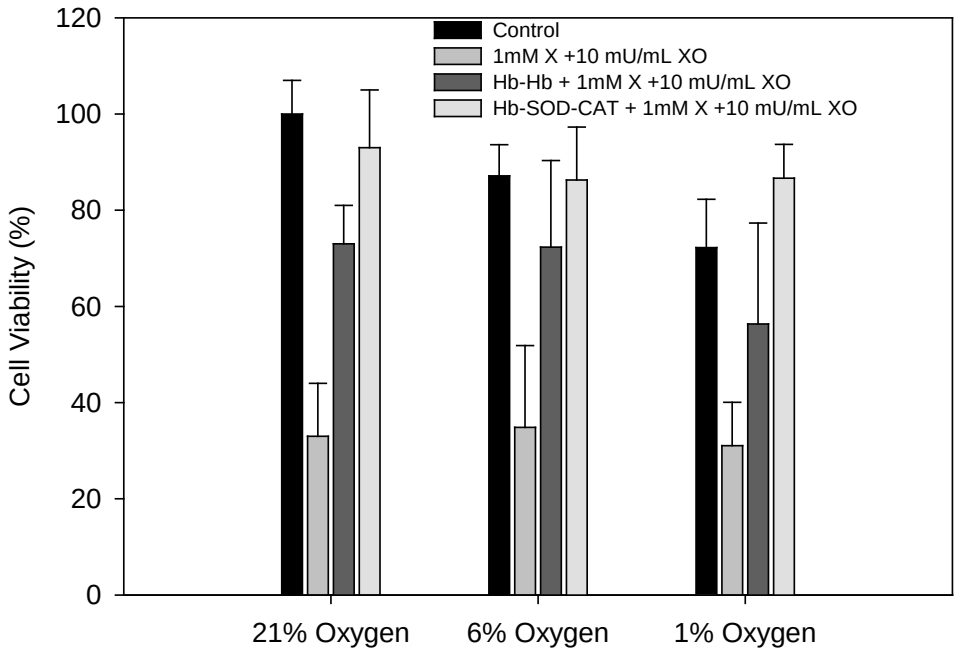

Figure 4.8: Relative cell viability of RINm5F cells incubated with hemoglobin conjugates and 1mM xanthine and 10mU/mL xanthine oxidase at 21%, 6% or 1% oxygen for 24h at 37 °C.

observed in cell killing when compared between control cells in 21% oxygen and cells treated with superoxide anion ($p < 0.001$) at partial pressures (21%, 6%, and 1%) of oxygen. In addition, superoxide anion-treated cells incubated with Hb-Hb without antioxidant enzymes ($p < 0.05$) also showed differences at 1% oxygen, indicating the inability of Hb-Hb alone to protect cells in severe combined free-radical and 1% hypoxic stress.

4.4.4 Insulin release by pancreatic beta islets

Insulin release from pancreatic islets challenged with 1mM hydrogen peroxide or superoxide anion and incubated in 21%, 6%, and 1% oxygen for 24h

followed by a 25mM glucose challenge test showed significant retention of the insulin release function for islets incubated with hemoglobin conjugates containing antioxidant enzyme (Hb-SOD-CAT), when normalized to control islets (21% oxygen) and compared to islets incubated with hemoglobin conjugates without antioxidant enzymes (Hb-Hb).

In 21% oxygen, islets only (no conjugates) challenged with 1mM hydrogen peroxide showed reduced insulin release of 21 ± 31%, islets incubated with Hb-Hb showed insulin release of 29 ± 26%, and islets incubated with Hb-SOD-CAT released 84 ± 29% insulin compared to control healthy islets (Figure 4.9). When exposed to 1mM hydrogen peroxide at 6% partial oxygen pressure, insulin release was 32 ± 13% for islets (no conjugates) and 33 ± 35% for islets and Hb-Hb, but was maintained at 86 ± 21% for islets with Hb-SOD-CAT when normalized to control islets cultured at 21% oxygen. For 1mM hydrogen peroxide exposure at 1% partial oxygen partial pressure, insulin secretion was 31 ± 10% for islets (no conjugates), 39 ± 10% for islets with Hb-Hb, and 86 ± 29% for islets incubated with Hb-SOD-CAT, when normalized to control islets cultured at 21% oxygen. Statistical significance was observed between control islets (no conjugates, 21% oxygen) and 1mM hydrogen peroxide-incubated islets ($p < 0.005$) and peroxide with Hb-Hb incubated islets ($p < 0.01$), suggesting substantial loss of insulin-secreting capability of islets at all partial pressures (21%, 6%, and 1%) of oxygen. In addition, the insulin-secretion differences were insignificant ($p > 0.4$) with Hb-SOD-CAT-incubated islets and show the direct and/or indirect protection of islets by antioxidant enzymes.

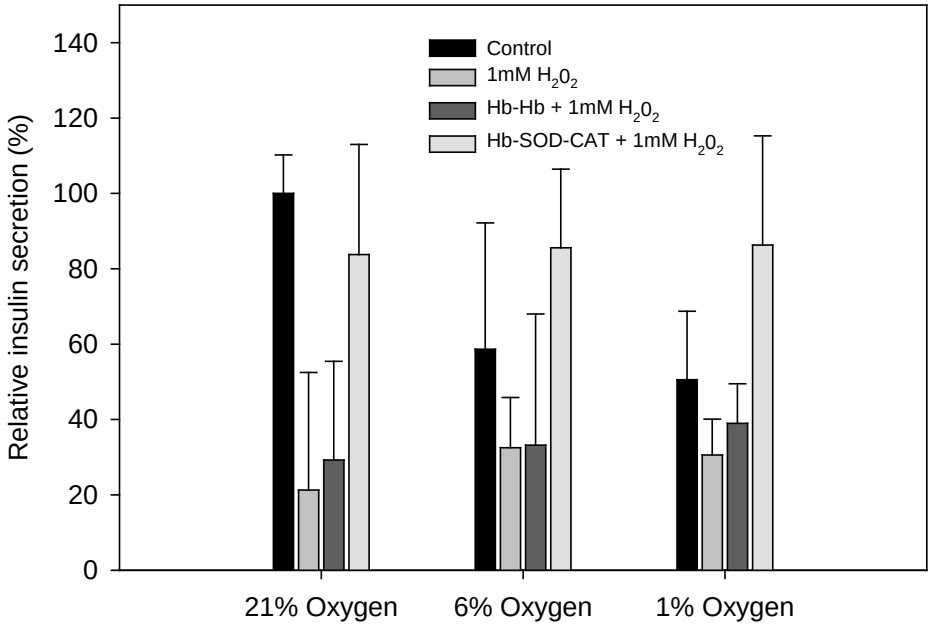

Figure 4.9: Relative insulin secretion from isolated rat pancreatic islets incubated with hemoglobin conjugates and 1mM H_2O_2 at 21%, 6% or 1% oxygen for 24h at 37 °C.

Superoxide anion-challenged islets (1mM xanthine and 10mU/mL xanthine oxidase) had reduced insulin secretion to 44 ± 22%, 23 ± 7%, and 21 ± 26% with superoxide anion only; and with superoxide anion and Hb-Hb incubation, reduced insulin release to 41 ± 36%, 30 ± 5%, and 22 ± 33%. Insulin release was maintained at 83 ± 22%, 77 ± 31%, and 94 ± 20% with superoxide anion and Hb-SOD-CAT incubation at 21%, 6%, and 1% partial oxygen pressures, respectively, and 37 °C for 24h challenge (Figure 4.10). Insulin secretion levels were statistically insignificant ($p > 0.2$) between islets (no conjugates, 21% oxygen) and islets challenged with superoxide anion and

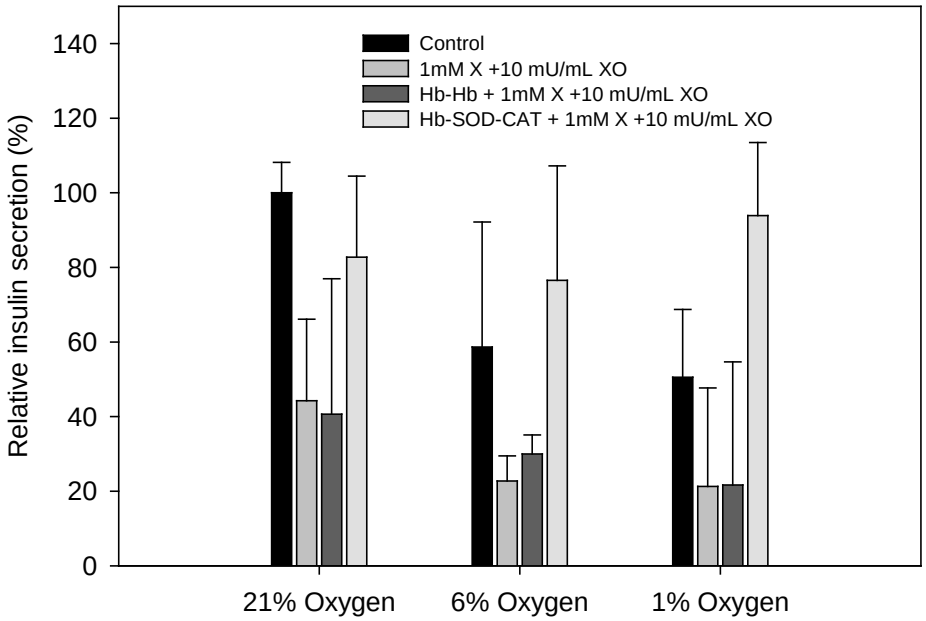

Figure 4.10: Relative insulin secretion from isolated rat pancreatic islets incubated with hemoglobin conjugates and 1mM xanthine and 10mU/mL xanthine oxidase at 21%, 6% or 1% oxygen for 24h at 37 °C.

incubated with Hb-SOD-CAT, showing the protection from free radical and hypoxic damage at tested partial oxygen pressures (21%, 6%, and 1%). The insulin secretions were statistically significant for islets challenged with superoxide anion ($p < 0.01$), and superoxide and Hb-Hb incubated islets ($p < 0.01$) at partial pressures (21%, 6%, and 1%) of oxygen.

4.4.5 Qualitative confocal microscopic image analysis of pancreatic beta cells

Control pancreatic islets incubated in 21% oxygen show high uptake of AO and no uptake of PI, indicating complete cell viability (Figure 4.11). Islets

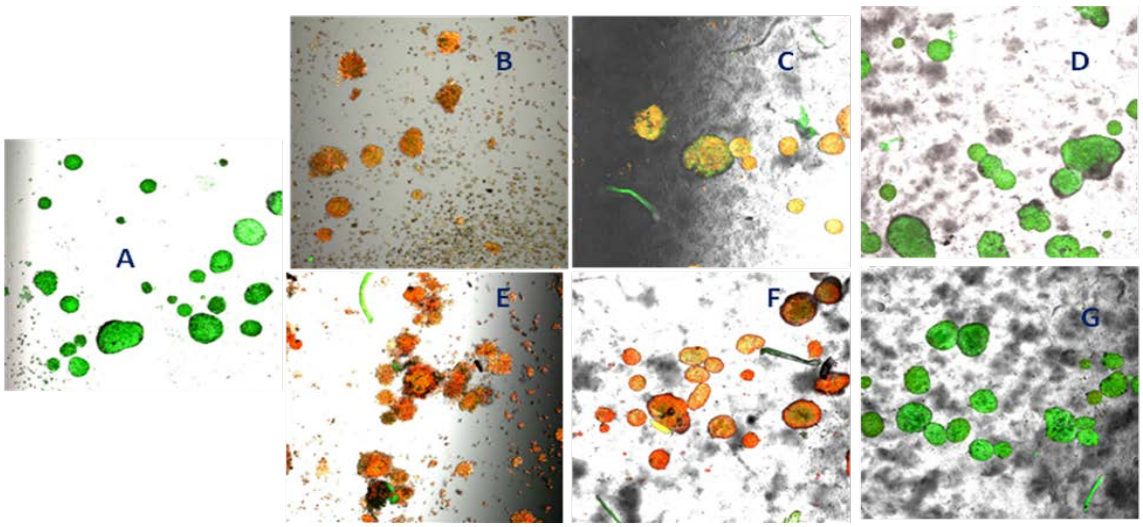

Figure 4.11: Confocal images of isolated rat pancreatic islets incubated with hemoglobin conjugates and either 1mM H_2O_2 or 1mM xanthine (X) and 10mU/mL xanthine oxidase (XO) at 21% oxygen for 24h at 37 °C.
(A) Control 1% O_2 (B) 1mM H_2O_2 (C) 1mM H_2O_2 + Hb-Hb (D) 1mM H_2O_2 + Hb-SOD-CAT (E) 1mM X and 10mU/mL XO (F) 1mM X and 10mU/mL XO + Hb-Hb (G) 1mM X and 10mU/mL XO +Hb-SOD-CAT (original magnification × 100).

incubated in 6% or 1% oxygen showed higher uptake of AO with little uptake of PI, indicating the susceptibility of islets to hypoxic stress (Figure 4.12 and 4.13). Islets only (no conjugates) and islets incubated with 0.1mM Hb-Hb and challenged with 1mM hydrogen peroxide had shown high uptake of PI with minimal uptake of AO at partial oxygen pressures (21%, 6% or 1% oxygen) indicating islet damage. Islets that were incubated with 0.1mM Hb-SOD-CAT and challenged with 1mM hydrogen peroxide showed high uptake of AO and minimal uptake of PI, indicating that there was significant islet protection from combined stress conferred by the hemoglobin and antioxidant enzymes in Hb-SOD-CAT in tested partial oxygen pressures.

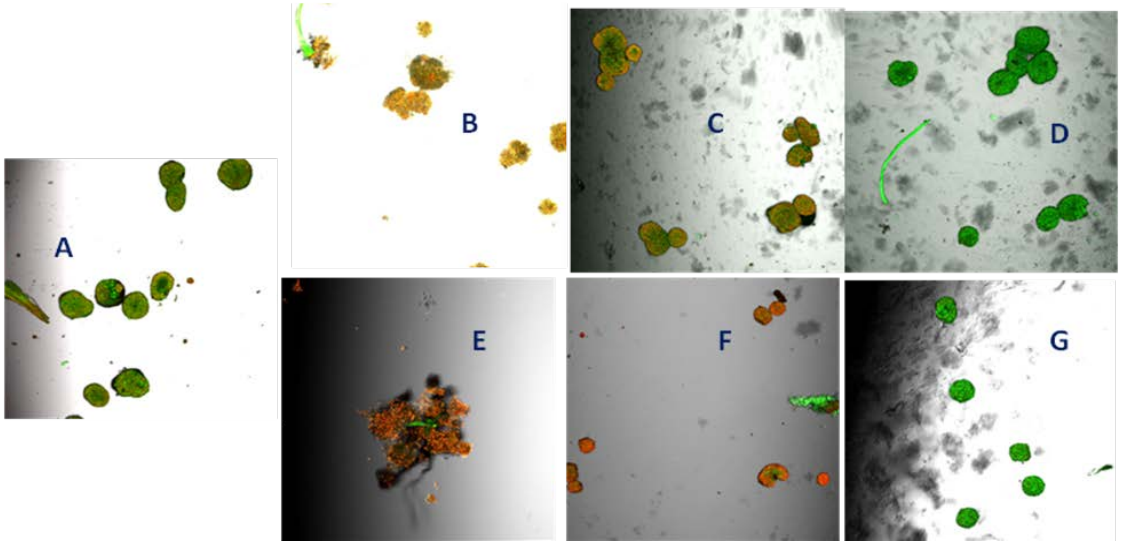

Figure 4.12: Confocal images of isolated rat pancreatic islets incubated with hemoglobin conjugates and either 1mM H_2O_2 or 1mM xanthine (X) and 10mU/mL xanthine oxidase (XO) at 6% oxygen for 24h at 37 °C.
(A) Control 1% O_2 (B) 1mM H_2O_2 (C) 1mM H_2O_2 + Hb-Hb (D) 1mM H_2O_2 + Hb-SOD-CAT (E) 1mM X and 10mU/mL XO (F) 1mM X and 10mU/mL XO + Hb-Hb (G) 1mM X and 10mU/mL XO +Hb-SOD-CAT (original magnification × 100).

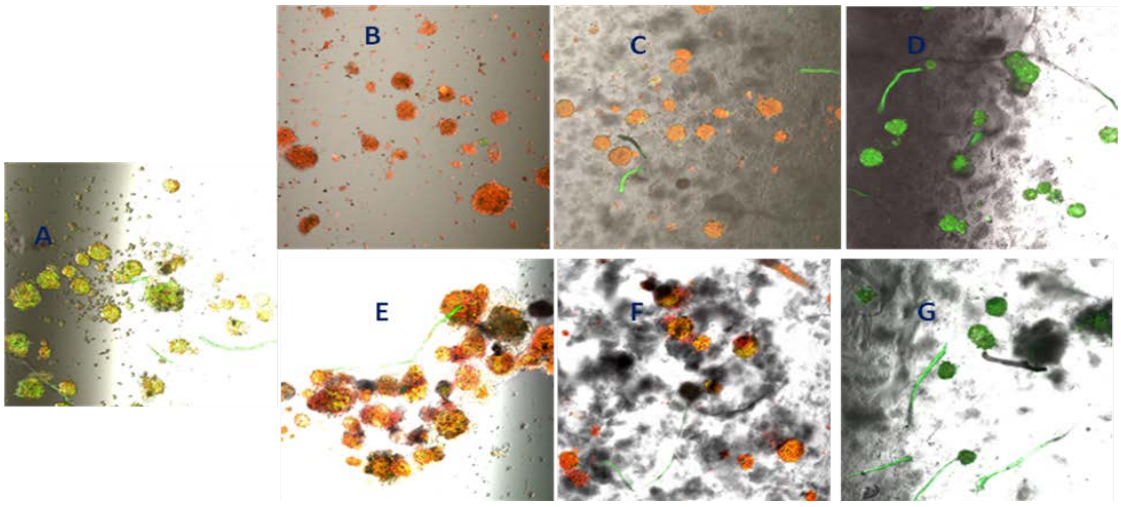

Figure 4.13: Confocal images of isolated rat pancreatic islets incubated with hemoglobin conjugates and either 1mM H_2O_2 or 1mM xanthine (X) and 10mU/mL xanthine oxidase (XO) at 21%, %6 or 1% oxygen for 24h at 37 °C.
(A) Control 1% O_2 (B) 1mM H_2O_2 (C) 1mM H_2O_2 + Hb-Hb (D) 1mM H_2O_2 + Hb-SOD-CAT (E) 1mM X and 10mU/mL XO (F) 1mM X and 10mU/mL XO + Hb-Hb (G) 1mM X and 10mU/mL XO +Hb-SOD-CAT (original magnification × 100).

Islets only (no conjugates) that were challenged with superoxide anion (1mM xanthine and 10mU/mL of xanthine oxidase) showed increased uptake of PI and minimal or decreased uptake of AO, indicating there was cell damage caused by the superoxide anion. Islets incubated with 0.1mM Hb-Hb and exposed to superoxide anion also showed uptake of PI and had qualitative images similar to superoxide-challenged islets only (no conjugates). However, when islets were incubated with Hb-SOD-CAT and exposed to superoxide anion, PI uptake was minimal and AO uptake was high, indicating that islets were well-protected from the combined stresses of superoxide anion damage and hypoxia over 24h in 21%, 6%, and 1% oxygen.

4.5 Discussion

Many factors hamper beta-cell function before and after islet transplantation (*24-27*). Hypoxic and oxidative stress plays an important role, and research reports suggest the need for protection of beta-cells by oxygenating (*28*) and using antioxidant enzymes (*9*). Even before transplantation, islets are subjected to metabolic adaptation because of isolation (*29*). Hemoglobin, the oxygen carrier, is the most important component of the conjugate system that needs protection from free-radical stress if it is going to extend the oxygen release and protect isolated beta-cells during hypoxic stress. Visible absorbance spectrum results on PEG-conjugated hemoglobins (Hb:PEG 1:10 molar ratio) had shown a substantial decrease of available hemoglobin and showed increased absorbance peak values at 630 nm, which represents the increased

non-oxygen-carrying methemoglobin formation in Hb-Hb cross-linked conjugates than conjugates with antioxidant enzymes (Hb-SOD-CAT). The results demonstrate with both the oxidants (superoxide anion and peroxide) that free radical damaging effects on hemoglobin are more rapid and prominent for the oxidant concentrations used in the experiment than the differences caused by oxygen partial pressures (21, 6%, and 1%) at 37 $^{\circ}$C and within 24h. Methemoglobin levels were higher in Hb-Hb conjugates without the antioxidant enzymes SOD and CAT, thus the damage caused by hydrogen peroxide and superoxide anion on hemoglobin. Although methemoglobin is formed in Hb-SOD-CAT oxidant-incubated conjugates, results show that the levels are approximately much lower when antioxidant enzymes are present and emphasize the need for their addition to inhibit the conversion of hemoglobin to methemoglobin. If necessary, this amount of methemoglobin may be further decreased by increasing the amount of antioxidant enzymes in the cross-linked conjugates.

It was reported that H_2O_2 concentration in normal human plasma ranges between 4-5 µM and increased in the inflammatory conditions (*30, 31*). In *in vivo* transplantation sites, the oxidant conditions on hemoglobin may not be as harsh as the *in vitro* experiments. Studies from another ascorbate-glutathione system also strongly suggested the need for an antioxidant system to inhibit the pro-oxidant effect of acellular hemoglobins (*12*). Previously, it was also reported that higher methemoglobin levels are formed in hemoglobin systems as the partial oxygen levels are decreased (*32*). On the contrary, the effect of different oxygen

levels (21%, 6%, and 1%) in the tested incubator seems to play an insignificant role when compared to the amount of the hydrogen peroxide or superoxide anion levels for 24h and at 37 °C incubation. However, in these studies, the effect may also be confounded by the oxidants' effects, or the effect by partial oxygen pressures may become more prominent in *in vivo* experiments longer than 24h. These observed results clearly show the rationale for inclusion of antioxidant enzymes in the conjugates for protection of hemoglobin from both hypoxia-induced and exogenous free radical stresses.

The cell viability of RINm5F cells challenged with oxidants measured the active mitochondrial MTT conversion to formazan (purple-shaped crystals). Results in RINm5F cells had demonstrated that cross-linked Hb-SOD-CAT offered higher cell protection in terms of percentage of relative cell viability than an Hb-Hb conjugate without the antioxidant enzymes from both combined oxygen tension (21%, 6%, and 1% oxygen) and peroxide or superoxide anion free radical stress. Higher cell viability observed in RINm5F cells incubated with Hb-SOD-CAT may be attributed primarily to the better protection of hemoglobin from free radical stress and thus the extended protection of cells from both hypoxia and free radical damages. Protection of RINm5F cells even with Hb-Hb conjugates incubated with oxidants may be oxidants had attacked hemoglobin initially than RINm5F cells. The cells are spared from hypoxic and free radical stress initially and as hemoglobin is degraded, hypoxia and further reactive active species might have acted and resulted on RINm5F cell death at latter stages. However, complete cell death in extended incubations beyond 24h studies can

be expected with Hb-Hb conjugates without antioxidant enzymes. Reduced intracellular ROS production with HB-SOD-CAT when compared to Hb-Hb seemed to be efficient in protecting the cells from the harmful effect of the oxidants. These results complement the viability results in RINm5F cells.

When cells are subjected to external stresses, cell death is the end point, and many metabolic functions may be shut down long before the cell death as a defense mechanism to protect cells from eventual cell death (*33*). Functions such as insulin release are affected highly in beta cells in hypoxic and free radical conditions (*34-36*). The results show that Hb-SOD-CAT had either protected or enhanced the insulin release by beta cells at tested partial oxygen pressures when challenged with free radical generating peroxide or superoxide anion in hypoxic conditions when normalized to the control islets in 21% oxygen. The results show the beneficial effect in restoring the metabolic function of islets from either combined peroxide challenge or superoxide anion challenge in hypoxia and retention or enhancement of insulin release function by the hemoglobin conjugated with antioxidant enzyme (Hb-SOD-CAT).

The combination of the fluorescent dyes AO (binds to DNA in viable cells) and PI (only permeable into apoptotic cells) also helped to qualitatively distinguish the cell death and viability of beta-cells. Isolated pancreatic beta-cells incubated with hydrogen peroxide and superoxide anion and by Hb-Hb conjugates without antioxidant enzymes in different partial oxygen pressures did not completely protect islets from free radical and hypoxic damage and thus emphasize the need for the addition of antioxidant enzymes into Hb-Hb cross-

linking. Hb-SOD-CAT conjugate incubated beta cells had shown minimal uptake of PI and the primary reason may be attributed to effective higher oxygen supply from the conjugated hemoglobins in severe hypoxic environments and free radical stress when antioxidant enzymes are used for the direct protection of Hb damage and beta- cells. These results strongly suggest that Hb-SOD-CAT may be an appropriate strategy to prevent hypoxia-induced graft failure in conditions such as encapsulated islet transplantation and ischemia re-perfusion injury (*37*). The hemoglobin function in a hypoxic environment is more effective in supplying oxygen when antioxidant enzymes protect Hb damage from free radical environment and thus shows the requirement for (Hb-SOD-CAT) design.

4.6. Conclusion

In this work, it is demonstrated the effectiveness of the Hb-SOD-CAT conjugate system for overall function of beta-cells similar to transplantation conditions. Cells incubated with only Hb-Hb in hypoxic conditions and free radical environment without antioxidant enzymes showed increased cell death in combined hypoxic and free radical environment. The hemoglobin function in a hypoxic environment is more effective in supplying oxygen when antioxidant enzymes protect Hb damage. These results strongly demonstrate the beneficial effect of adding SOD and CAT for the protection of hemoglobin and/or beta-cells against hypoxic and oxidative stresses and thus the inhibition of methemoglobin formation to extend oxygen supply.

4.7 Acknowledgements

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4.8 References

(1) Meloche, R. M. (2007) Transplantation for the treatment of type 1 diabetes. *World J Gastroenterol* 13, 6347-55.

(2) Beck, J., Angus, R., Madsen, B., Britt, D., Vernon, B., and Nguyen, K. T. (2007) Islet encapsulation: strategies to enhance islet cell functions. *Tissue Eng* 13, 589-99.

(3) Giraldo, J. A., Weaver, J. D., and Stabler, C. L. (2010) Tissue engineering approaches to enhancing clinical islet transplantation through tissue engineering strategies. *J Diabetes Sci Technol* 4, 1238-47.

(4) Bae, Y. H. (2004) Caged pancreatic islet for IDDM. *Yonsei Med J* 45 Suppl, 56-60.

(5) Rao, P., Maeda, H., Yutong, X., Yamamoto, M., Hirose, N., and Sasaguri, S. (2005) Protective effect of a radical scavenger, MCI-186 on islet cell damages induced by oxidative stress. *Transplant Proc* 37, 3457-8.

(6) Linn, T., Schmitz, J., Hauck-Schmalenberger, I., Lai, Y., Bretzel, R. G., Brandhorst, H., and Brandhorst, D. (2006) Ischaemia is linked to inflammation and induction of angiogenesis in pancreatic islets. *Clin Exp Immunol* 144, 179-87.

(7) Ko, S. H., Ryu, G. R., Kim, S., Ahn, Y. B., Yoon, K. H., Kaneto, H., Ha, H., Kim, Y. S., and Song, K. H. (2008) Inducible nitric oxide synthase-nitric oxide plays an important role in acute and severe hypoxic injury to pancreatic beta cells. *Transplantation* 85, 323-30.

(8) Carlsson, P. O., Liss, P., Andersson, A., and Jansson, L. (1998) Measurements of oxygen tension in native and transplanted rat pancreatic islets. *Diabetes* 47, 1027-32.

(9) Mohseni Salehi Monfared, S. S., Larijani, B., and Abdollahi, M. (2009) Islet transplantation and antioxidant management: a comprehensive review. *World J Gastroenterol* 15, 1153-61.

(10) Chae, S. Y., Kim, Y. Y., Kim, S. W., and Bae, Y. H. (2004) Prolonged glucose normalization of streptozotocin-induced diabetic mice by transplantation of rat islets coencapsulated with crosslinked hemoglobin. *Transplantation* 78, 392-7.

(11) Chae, S. Y., Kim, S. W., and Bae, Y. H. (2002) Effect of cross-linked hemoglobin on functionality and viability of microencapsulated pancreatic islets. *Tissue Eng* 8, 379-94.

(12) Simoni, J., Villanueva-Meyer, J., Simoni, G., Moeller, J. F., and Wesson, D. E. (2009) Control of oxidative reactions of hemoglobin in the design of blood substitutes: role of the ascorbate-glutathione antioxidant system. *Artif Organs* 33, 115-26.

(13) Winterbourn, C. C. (1990) Oxidative reactions of hemoglobin. *Methods Enzymol* 186, 265-72.

(14) Vallelian, F., Pimenova, T., Pereira, C. P., Abraham, B., Mikolajczyk, M. G., Schoedon, G., Zenobi, R., Alayash, A. I., Buehler, P. W., and Schaer, D. J. (2008) The reaction of hydrogen peroxide with hemoglobin induces extensive alpha-globin crosslinking and impairs the interaction of hemoglobin with endogenous scavenger pathways. *Free Radic Biol Med* 45, 1150-8.

(15) Nadithe, V., and Bae, Y. H. (2010) Synthesis and characterization of hemoglobin conjugates with antioxidant enzymes via poly(ethylene glycol) cross-linker (Hb-SOD-CAT) for protection from free radical stress. *Int J Biol Macromol* 47, 603-13.

(16) Nadithe, V., and Bae, Y. H. (2010) Hemoglobin conjugates with antioxidant enzymes (Hb-SOD-CAT) via poly(ethylene glycol) crosslinker for protection of pancreatic beta RINm5F cells in hypoxia. *Tissue Eng* Accepted.

(17) Patton, J. N., and Palmer, A. F. (2005) Photopolymerization of bovine hemoglobin entrapped nanoscale hydrogel particles within liposomal reactors for use as an artificial blood substitute. *Biomacromolecules* 6, 414-24.

(18) Rodrigues, J. V., and Gomes, C. M. (2010) Enhanced superoxide and hydrogen peroxide detection in biological assays. *Free Radic Biol Med* 49, 61-6.

(19) von Sonntag, C. (2008) Advanced oxidation processes: mechanistic aspects. *Water Sci Technol* 58, 1015-21.

(20) Tulsawani, R., Kelly, L. S., Fatma, N., Chhunchha, B., Kubo, E., Kumar, A., and Singh, D. P. (2010) Neuroprotective effect of peroxiredoxin 6 against hypoxia-induced retinal ganglion cell damage. *BMC Neurosci* 11, 125.

(21) Hayon, T., Dvilansky, A., Shpilberg, O., and Nathan, I. (2003) Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. *Leuk Lymphoma* 44, 1957-62.

(22) Kang, H. C., and Bae, Y. H. (2006) Polymeric gene transfection on insulin-secreting cells: sulfonylurea receptor-mediation and transfection medium effect. *Pharm Res* 23, 1797-808.

(23) Kim, Y. Y., Chae, S. Y., Kim, S., Byun, Y., and Bae, Y. H. (2005) Improved phenotype of rat islets in a macrocapsule by co-encapsulation with cross-linked Hb. *J Biomater Sci Polym Ed* 16, 1521-35.

(24) Watson, D., and Loweth, A. C. (2009) Oxidative and nitrosative stress in beta-cell apoptosis: their contribution to beta-cell loss in type 1 diabetes mellitus. *Br J Biomed Sci* 66, 208-15.

(25) Vaithilingam, V., Sundaram, G., and Tuch, B. E. (2008) Islet cell transplantation. *Curr Opin Organ Transplant* 13, 633-8.

(26) Teramura, Y., and Iwata, H. (2010) Bioartificial pancreas microencapsulation and conformal coating of islet of Langerhans. *Adv Drug Deliv Rev* 62, 827-40.

(27) Xu, J., Long, Y. S., Gozal, D., and Epstein, P. N. (2009) Beta-cell death and proliferation after intermittent hypoxia: role of oxidative stress. *Free Radic Biol Med* 46, 783-90.

(28) Sakai, T., Li, S., Kuroda, Y., Tanioka, Y., Fujino, Y., and Suzuki, Y. (2011) Oxygenation of the portal vein by intraperitoneal administration of oxygenated perfluorochemical improves the engraftment and function of intraportally transplanted islets. *Pancreas* 40, 403-9.

(29) Gerbitz, K. D., Gempel, K., and Brdiczka, D. (1996) Mitochondria and diabetes. Genetic, biochemical, and clinical implications of the cellular energy circuit. *Diabetes* 45, 113-26.

(30) Bragt, P. C., and Bonta, I. L. (1980) Oxidant stress during inflammation: anti-inflammatory effects of antioxidants. *Agents Actions* 10, 536-9.

(31) Nagababu, E., and Rifkind, J. M. (2000) Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation. *Biochemistry* 39, 12503-11.

(32) Takeoka, S., Sakai, H., Kose, T., Mano, Y., Seino, Y., Nishide, H., and Tsuchida, E. (1997) Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. *Bioconjug Chem* 8, 539-44.

(33) Saraste, A., and Pulkki, K. (2000) Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res* 45, 528-37.

(34) de Groot, M., Schuurs, T. A., Keizer, P. P., Fekken, S., Leuvenink, H. G., and van Schilfgaarde, R. (2003) Response of encapsulated rat pancreatic islets to hypoxia. *Cell Transplant* 12, 867-75.

(35) Dionne, K. E., Colton, C. K., and Yarmush, M. L. (1993) Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. *Diabetes* 42, 12-21.

(36) Pfeiffer, R. M., Goldin, L. R., Chatterjee, N., Daugherty, S., Hemminki, K., Pee, D., X, L. I., and Gail, M. H. (2004) Methods for testing familial aggregation of diseases in population-based samples: application to Hodgkin lymphoma in Swedish registry data. *Ann Hum Genet* 68, 498-508.

(37) Chang, T. M. (2010) Blood replacement with nanobiotechnologically engineered hemoglobin and hemoglobin nanocapsules. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2, 418-30.

5. CONCLUSIONS AND FUTURE WORK

5.1 General conclusions

The biohybrid artificial pancreas approach employing encapsulated islets with biocompatible polymers offers an immunoprotective environment that could overcome some limitations of the current antidiabetic therapy. Numerous research studies reported that the separation of islets renders them susceptible to hypoxic and free-radical stress, causing enhanced apoptosis at the site of transplantation. Newer technologies such as stem cells may provide the solutions for the islet scarcity. The critical problems associated with hypoxia, which reduces insulin secretion, need to be addressed to successfully advance the islet transplantation and to achieve clinical success. The research project focused on solving the primary problem of hypoxia-associated death in islets by using low p50 hemoglobin (Hb) and of free-radical stress (hypoxia associated) by using the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). The ultimate goal of the work is to enhance the cell viability of beta-cells to potentially provide a long-term continuous secretion of insulin after transplantation for advancement toward a biohybrid artificial pancreas.

The first part of the study focused on cross-linking Hb with SOD and CAT enzymes to stabilize the proteins and increase the molecular size of conjugates intended for the retention in the microcapsules to prevent diffusional loss. A suitable polymer was chosen to reduce the conversion of hemoglobin to methemoglobin during cross-linking. For the long-term protection of Hb from oxidative stress during storage or at the transplantation site, either by auto-oxidation or free radicals, appropriate antioxidant enzymes SOD and CAT were

cross-linked along with Hb. Retention of enzymatic activity was also confirmed after cross-linking the enzymes SOD and CAT with HB. The results proved that Hb can be significantly protected by using the current method of PEG cross-linking. The conversion of hemoglobin to methmoglobin by oxidative stress was also verified and found to be minimal by the cross-linking approach. The above results helped to predict that Hb may extend oxygen delivery function in scenarios that involve free-radical stress. Different cross-linking ratios of PEG/Hb were screened, and the ratio 1:10 was found to be optimal for further studies. Overall, the use of antioxidant enzymes in cross-linking introduced a new type of PEG-modified hemoglobin oxygen carrier.

The second part focused on whether low p50 characteristics of Hb helps in simulated situations of hypoxia-induced stress. The protective effect evaluated on pancreatic beta cells (RINm5F) from hypoxia (6%, 3%, and 1% oxygen) by an MTT assay and confocal microscopy showed Hb conjugates with antioxidant enzymes offered significant protection ($p < 0.01$, increased viability ~ 80%) from hypoxia compared to control cells in 1% oxygen. The higher protection of RINm5F cells with Hb conjugates when compared to free Hb may be primarily because of the differences in their p50 characteristics. This work provided a polymer-based cross-linking approach to lower the p50 of hemoglobin for optimization of O_2 carriers, intended for therapeutic or prophylactic benefits from hypoxia and ischemic conditions. This study also demonstrated a general design approach for protecting isolated cells or tissues from combined hypoxia-induced oxidative and hypoxic stress.The last part of the work demonstrated the

usefulness of Hb-SOD-CAT for the overall function of beta-cells when exposed to conditions mimicking islet transplantation.

Hemoglobin is the most important component of the conjugate systems and needs first-hand protection from free radical stress for it to extend the protection function on cells by oxygen release. Visible absorption spectrum results showed a substantial decrease in non-oxygen-carrying methemoglobin formation with antioxidant enzymes (Hb-SOD-CAT) and thus the protection of hemoglobin in a harsh free radical environment. The results demonstrated that free-radical damage by both superoxide anion and hydrogen peroxide is more rapid and prominent on Hb than the differences in oxygen partial pressures.

The cell viability results on RINm5F cells demonstrated that cross-linked Hb-SOD-CAT offered better cell protection in terms of relative cell viability from both combined oxygen tension and peroxide or superoxide anion free-radical stress. Confocal microscopy helped to qualitatively distinguish the protection on pancreatic beta islets in the combined hypoxic and oxidative conditions, and the results showed that Hb-SOD-CAT offered better cell protection. Insulin release by beta cells at all partial oxygen pressures was retained when beta cells were subjected to combined oxidative and hypoxic stresses and tested by glucose-induced insulin secretion. Reduced intracellular ROS production with HB-SOD-CAT also has demonstrated the effectiveness of conjugate systems in protecting the cells from oxidants.

These results show the potential of Hb-SOD-CAT in preventing the beta-cell dysfunction usually associated with free-radical and hypoxic stress

encountered during transplantation or for clinical situations such as organ preservation for transplantation.

5.2 Future work

5.2.1 Optimization of co-encapsulated cross-linked Hb-SOD-CAT and islets within microcapsules

This work will demonstrate whether islets and Hb-SOD-CAT can effectively be co-encapsulated into the microcapsules and the system can be functional with the islet post encapsulation. Ideally, a single islet in one microcapsule may function better. Depending upon whether a single islet or multiple islets are encapsulated, the amount of Hb-SOD-CAT in a single microcapsule needs to be optimized. Low amounts of Hb-SOD-CAT may not provide enough oxygen content to the islet. The compatibility between the capsule-forming material and Hb-SOD-CAT should also be tested. Incompatible capsule-forming materials may cause imperfections in the capsule morphologies, such as tapering ends of the capsules, which will create pores and thus not retain Hb-SOD-CAT and/or islets. These imperfections may also create entry into the capsule by other molecules or cells that may have damaging or immune-generating activity on islets and eventually may lead to partially encapsulated islet death. The entire capsule system (capsule material, islet, and Hb-SOD-CAT) should also be tested *in vitro* in hypoxia and/or hypoxia-induced free radical stimulated conditions. The results from these comprehensive experiments will

help in further selection and optimization of microcapsules for efficient islet delivery and thus the prolonged insulin secretion at transplantation site.

5.2.2 Transplantation studies in diabetic animal models

Optimized microcapsules should be further tested in the Type 1 diabetic models. Type 1 diabetic animal models are widely used for studying the effectiveness of the antidiabetic drug therapy. The first is the chemically induced diabetic rat model, in which diabetes is induced by streptozotocin (STZ). The second is the spontaneous autoimmune type with nonobese diabetic (NOD) mice, in which diabetes is induced by viral infection. Even though these two models differ only in the way of generating a experimental diabetic model, the results will help researchers understand how efficiently the proposed approach works in the preclinical stage.

Microencapsulated islets with co-encapsulated Hb-SOD-CAT should be transplanted at different common islet transplantation sites (peritoneal, portal, etc.) by measuring the partial oxygen pressures. The effectiveness of the whole approach should be tested by frequently collecting blood samples and measuring blood glucose levels. The *in vivo* glucose level estimation will help in understanding the ability of the encapsulated islets and the number islets needed to achieve normoglycemia in the diabetic preclinical model. The effectiveness and protection of islets at the transplantation sites can be further tested by retrieving microcapsules and challenging with glucose for the islet ability to release insulin. This will help researchers understand the functionally of the islets

(quality) before and after transplantation and thus the robustness of islet protection in the capsule. Hemoglobin and islets quality should also be assessed in decapsulating conditions. The protection of the hemoglobin at the actual transplantation site will also help the usefulness of antioxidant enzymes in protecting the hemoglobin from free radical and auto-oxidation.